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AN ETIOLOGIC STUDY OF HODGKIN'S DISEASE.

SECOND NOTE.*

BY C. H. BUNTING, M.D., AND J. L. YATES, M.D.

(From the Pathological Laboratory of the University of Wisconsin.)

In a preliminary note recently published,[1] we reported that by repeated injection of the diphtheroid organism cultivated by us from cases of Hodgkin's disease, there had been produced in monkeys lesions of the lymph-nodes showing all the essential features of early Hodgkin's disease in man. Up to that time we had been unable to demonstrate that the organism could survive in the monkey for any great length of time, and therefore we felt that we could not assert that we had produced Hodgkin's disease in the monkey, or that we had even demonstrated any great pathogenicity of the diphtheroid organism for that species.

Since making that report, however, the course of our experimental work has demonstrated fully the pathogenicity of the culture we were using, and has further shown that the virulence of the organism to the monkey may easily be increased even to the point of producing death of the animal after a relatively acute illness. While the histologic picture of the enlarged lymph-nodes of the monkey taken three months after the successful inoculation, leaves no question as to the relation of the lesion to that of human Hodgkin's disease of the same duration, the great difficulty seems to be to secure infection and at the same time to avoid so great virulence as to produce extensive necrosis and softening and even suppuration. The working space between these two limits seems very narrow.

Extensive necrosis and leukocytic infiltration of the glands may

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* This work has been aided by a grant from The Rockefeller Institute for Medical Research.

[1] Bunting, C. H., and Yates, J. L.: An Etiologic Study of Hodgkin's Disease, THE JOURNAL A. M. A., Nov. 15, 1913, p. 1803.

seem foreign to the usual chronic picture of the lymph-nodes in Hodgkin's disease, yet a recent clinical case has demonstrated that even in man the virulence of the organism may be such as to lead to these features. With an apparent duration of six months, there is in this case marked involvement of cervical, axillary and mediastinal glands, febrile reaction and leukocytosis (44,000). While the excised glands show all the elements of well-developed Hodgkin's disease, there are, in addition, extensive areas of necrosis, softening and leukocytic infiltration. Yet culturally, the diphtheroid organism, which was obtained from both cervical and axillary glands, was the only organism to grow.

At present, our results indicate that the survival of an animal for the requisite length of time is all that is needed for a demonstration of the chronic lymph-node picture seen in the well-developed case of Hodgkin's disease.

Thus, since our experiments demonstrate that the diphtheroid organism is pathogenic for the monkey, that it produces a progressive enlargement of the lymph-nodes, with lesions similar to those of Hodgkin's disease in man, and further that the blood-changes in the monkey are similar to those in man, we feel fully assured of the etiologic relationship of the diphtheroid organism (*Bacterium hodgkini*) to Hodgkin's disease.

The experiments which have led to this conclusion are in outline as follows:

Monkey 1.—Covered by previous note. Repeated injections with diphtheroid organism between April 19 and June 30, 1913, with production of a chronic lymphadenitis, characteristic of early Hodgkin's disease. July 6, excision of glands for histologic study. Implantation of portion of gland into right axilla of Monkey A3.

Death of Monkey 1 from intussusception occurred before infection was secured.

Monkey A3.—Female rhesus.

July 6, 1913, lymph-nodes all small, shot-like. Under ether, tissue from Monkey 1, made up of parts of three lymph-nodes, introduced into axillary space. Fascia and skin closed by separate sutures.

July 9, skin wound slightly pulled apart, but clean. Fascial sutures holding well.

July 19, wound cleanly healed. A group of enlarged glands palpable in right axilla.

August 4, three enlarged glands still palpable in right axilla.

September 24, animal found dead, having died during night after gradual decline.

Post-mortem examination showed in right axilla a group of enlarged lymph-nodes, from 10 to 15 mm. in diameter, with softened areas from which, on incision, a thick puriform necrotic material was expressed. Extending upward from these nodes was a suppurative process reaching to the highest point in the axilla and involving the chest wall. The organs showed multiple metastatic abscesses in lung, heart, liver and pancreas. A hyperemic splenic tumor and cloudy swelling of the viscera were present.

Histologically, while the areas of necrosis and leukocytic infiltration were the most prominent feature of the picture in the adjacent parts of the nodes, there were all the elements of the Hodgkin's picture: distortion and disappearance of architecture; great proliferation of endothelioid cells, with marked development of endothelioid giant-cells, in some places with lobed nuclei, and proliferation of fibroblasts with both fine and patchy sclerosis. Eosinophil cells were found only occasionally (apparently due to exhaustion of the marrow as indicated by marrow sections).

The spleen showed a hyperemic tumor, pulp and sinuses being filled with red blood-cells. In addition the malpighian corpuscles showed a lesion distinct from the usual hyperplasia which results in a sharp outlining of the germinal center and a thick collar of lymphocytes. In this spleen, however, there was irregular and extensive proliferation of the endothelioid cells, with numerous mitotic figures present. Many of the cells were of the size and character of the endothelioid giant-cells in the lymph-nodes. There was also some fibroblastic proliferation in and adjacent to the corpuscles. A scattering of eosinophils was seen.

At the post-mortem examination, a culture was taken from the axillary lymph-nodes, and a pure culture of the diphtheroid organism was obtained. This grew very feebly for several generations but finally acquired greater power of growth. This culture was used in inoculating Monkey 3.

Monkey 3.—Female rhesus. Received April 11. Negative to von Pirquet test. April 26, first injection of two slants of a twenty-four-hour growth of culture X. F. A. subcutaneously into right thigh. Subsequent to this and up to August 17, ten injections of the same organism were made in the same location, and with the uniform result of the development of induration and glandular enlargement which subsided gradually. On one occasion, June 21, inoculation resulted in the formation of a subcutaneous abscess from which the diphtheroid organism was recovered in pure culture. This was subsequently used for inoculation in the hope that it might have acquired some increased virulence.

October 25, there were but shot-like glands in the right groin and it was felt that the animal was refractory to the culture.

October 31, inoculation was made into the right axilla of the scrapings in salt-solution of 3 slants of an almost invisible growth on egg-medium of the diphtheroid organism recovered from Monkey 3. The salt solution suspension appeared almost clear. There was no immediate sharp glandular reaction and one week later no sign of reaction along needle track.

December 6, there was a large, lobulated, indurated mass along pectoral edge and extending high in axilla, and consisting of enlarged glands distinct in outline but with connecting sclerosis. The lower glands were over 1 cm. in diameter, while high up in the axilla the glands appeared about 5 mm. in diameter. At the most prominent low point was a softened area with discharging sinus. The material discharged on pressure was a thick white material resembling broken-down glands.

LEUKOCYTE COUNTS IN MONKEY 3.

	Total	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
April 17	15,600	34.2	1.4	0.2	54	3.8	1.0	5.4
July 2	.....	22.4	7.8	0.2	57.4	5.8	0.2	6.2
July 12	19,000	25.2	4.2	0.2	60	4.2	0.4	5.8
October 31	.....	49.4	4.8	0.0	37.2	4.0	0.0	4.6
December 6	13,000	42	0.8	0.0	46	4.2	0.4	6.6
December 19	15,000	48	0.2	0.0	38.8	4.0	0.0	9.0
December 31*	.....	70.2	0.0	0.0	19.4	2.2	0.0	8.2

* December 31, 4 nucleated red cells to 500 leukocytes were found.

N = neutrophils; E, eosinophils; B, basophils; S. L., small lymphocytes; L. L., large lymphocytes; L. M., large mononuclears; and Tr., transitionals.

December 19, the wound was still discharging, and in the left axilla was an infected skin wound (apparently self-inoculation) with several enlarged lymph-nodes palpable above it.

December 31, a second softened area appeared in the right axilla about 3 cm. above the first.

Monkey 2.—Male rhesus, received from New York April 11. April 19 the first injection of a twenty-four-hour slant-growth of the culture X. F. A. was made into the right tonsillar region. When it was found that the enlargement of the cervical glands that resulted did not persist, the place of injection was changed to the cervical subcutaneous tissue, on account of the difficulty of reaching the tonsil without etherization. Three injections were made in the neck region. Then on June 30 the place of inoculation was changed to the right axilla. Here six injections were given, the dose being increased, until on August 17 four slants of a twenty-four-hour growth were injected. The intervening injections had resulted in the development of axillary induration, associated with glandular swelling which had persisted for about a week after the injection. After August 17 no injections were given. On September 15 a moderate enlargement of the right axillary glands was noted. This continued, and on November 1 there was in the right axilla a large, lobulated mass roughly 2.5 by 3 cm. in diameter, apparently made of five or six discrete glands with a certain amount of interglandular sclerosis.

November 23, under cocain, a gland was removed, cultures made, tissue fixed for study, and a portion of the gland implanted in Monkey A4.

LEUKOCYTE COUNTS IN MONKEY 2.

	Total	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
April 17	32,000	50.6	8.0	0.0	34.6	2.0	0.8	4.0
April 21	15,600	71.6	4.0	0.8	15.6	2.6	0.2	5.2
April 24	24,400	65.4	3.8	1.2	20.0	2.4	0.6	6.6
October 28	19,000	44.6	7.2	0.4	37.6	2.6	0.0	7.6
November 14	20,000	35.8	7.0	0.0	42.8	4.3	0.4	9.7
December 31	.....	46.3	2.9	0.4	33.7	5.6	0.4	10.7

One nucleated red blood-cell to 500 white blood-cells noted October 28; 1 November 14, and 2 to 500 December 31.

Macroscopically the gland showed large, opaque, softened, necrotic areas, surrounded by more translucent hyperplastic tissue. The softened material had the consistency neither of true pus nor of tuberculous caseation.

Histologically, the gland showed areas of necrosis infiltrated by leukocytes. There was some scarring of the gland. Outside of the necrotic areas the architecture of the gland was lost. The tissue was cellular, showing relatively few lymphocytes, but large numbers of endothelioid cells, many of which had very large nuclei, of the giant-cell type. Eosinophilic infiltration was present and marked in some areas. There was a periglandular sclerosis.

A pure culture of the diphtheroid organism was found in one serum-tube on which a piece of gland was planted.

December 6, the wound was perfectly healed, but an area of softening had developed at some distance from the wound and at the lowermost prominent point of the enlarged mass.

December 26, the skin was found necrotic at one point over this area, and a thick necrotic material exuded on pressure. A diphtheroid organism was found in smears from this material.

December 31, skin sinus appeared healed and dry. No further softening.

Monkey A4.—Large male rhesus.

November 23, right axilla shows only small shot-like glands high up. Under cocain anesthesia, a piece of gland from Monkey A2 was implanted in the right axilla. Fascia and skin were stitched separately.

December 6, wound was found perfectly healed. Several moderately enlarged glands were felt above site of implantation.

December 19, several enlarged glands in axilla formed a mass roughly 2 cm. in diameter.

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THE RELATION TO THE BLOOD OF THE VIRUS OF EPIDEMIC POLIOMYELITIS.*

BY PAUL F. CLARK, PH.D., FRANCIS R. FRASER, M.B., AND HAROLD L. AMOSS, M.D.

(From the Laboratories and Hospital of The Rockefeller Institute for Medical Research.)

Early in the history of experimental poliomyelitis Flexner and Lewis[1] succeeded in one instance in producing infection in a Macacus monkey through the intravenous injection of twenty-five cubic centimeters of the defibrinated blood obtained from a recently paralyzed monkey. When a smaller quantity (ten cubic centimeters) of the blood was injected into the circulation, or still smaller quantities were introduced intracerebrally, paralysis did not follow. Leiner and von Wiesner[2] at first reported only negative results from the injection of blood from both human and experimental cases of poliomyelitis; but later[3] they observed in one instance paralysis following the injection, partly intracerebral and partly intravenous, of defibrinated blood taken from a monkey on the third day of paralysis. Römer[4] and Landsteiner and Levaditi[5] also record only failure both with the blood of human and of experimental cases of the disease.

The subject of the relation to the blood of the virus of poliomyelitis is of more than theoretical interest, as it may have a bearing on the manner of transmission of the disease. On that account we have carried out a larger series of experiments in order to determine, as far as possible with the methods at present available, what this relation is.

The first series of inoculations was performed with blood taken

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* Received for publication, December 20, 1913.

[1] Flexner, S., and Lewis, P. A., *Jour. Exper. Med.*, 1910, xii, 227.

[2] Leiner, K., and von Wiesner, R., *Wien. klin. Wchnschr.*, 1909, xxii, 1698.

[3] Zappert, J., von Wiesner, R., and Leiner, K., Studien über die Heine-Medinsche Krankheit, Leipzig and Vienna, 1911, 171.

[4] Römer, P. H., *München. med. Wchnschr.*, 1910, lvii, 229.

[5] Landsteiner, K., and Levaditi, C., *Ann. de l'Inst. Pasteur,* 1910, xxiv, 833.

from human cases admitted to the Hospital of The Rockefeller Institute.

SURVIVING HUMAN CASES.

Case 1.—N. B. Age, 21 years. Onset Oct. 1, 1910. Condition: weakness and atrophy of muscles of right shoulder girdle and arm, atrophy of muscles of right thigh. Blood and spinal fluid normal. Blood collected 24/24.[6]

Oct. 25. 2 c.c. of defibrinated blood injected intracerebrally and 6 c.c. intraperitoneally into a *Macacus rhesus.* No symptoms of poliomyelitis developed.

Case 2.—J. H. Age, 12½ years. Onset Oct. 16, 1910. Condifion: partial paralysis of legs; temperature 101.6° F. Oct. 27. Increased globulin in spinal fluid. Blood collected 13/9.

Oct. 27. Immediately after collection 3 c.c. of the whole blood were injected intracerebrally and 6 c.c. intraperitoneally into a *Macacus rhesus.* No effects followed.

Case 3.—J. A. Age, 20 years. Onset July 14, 1911. Condition: complete paralysis of right and partial paralysis of left leg. Increased cells and globulin in spinal fluid. July 22. Blood collected and defibrinated 9/7.

July 22. 4 c.c. of the blood were injected intracerebrally and 10 c.c. intraperitoneally into a *Macacus rhesus.* Monkey remained well.

Case 4.—M. B. Age, 5 years. Onset Oct. 3, 1912. Condition: temperature 104.8° F.; right leg paralyzed; left leg weak; neck and back stiff; facial muscles on left side weak. Increased cells in spinal fluid. Oct. 8. Right arm paralyzed. Blood collected 6/3.

Oct. 8. The whole blood was immediately injected as follows: 2 c.c. intracerebrally, and 30 c.c. intraperitoneally into a *Macacus rhesus.* No ill effects followed.

Case 5.—A. J. Age, 10 years. Onset Oct. 13, 1912. Condition: tenderness in limbs and back; left arm weak; right shoulder and extensors of right arm paralyzed; intercostal muscles on both sides paralyzed. Oct. 23. Blood collected 11/7.

Oct. 23. Injected 30 c.c. of whole blood into the femoral vein of a *Macacus rhesus.* Monkey remained well.

Case 6.—H. O'C. Age, 3 years. Onset Nov. 1, 1912. Condition: temperature 101.8° F.; right leg paralyzed; intercostal muscles on left side weak; muscles of anterior abdominal wall and anal sphincters weak. Increased cells in spinal fluid. Nov. 5. Blood collected 5/4.

Nov. 5. 2 c.c. of whole blood were injected into the left sciatic nerve of a *Macacus rhesus.* No symptoms of poliomyelitis developed.

FATAL HUMAN CASES.

Case 7.—G. G. Age, 9½ years. Onset Aug. 23, 1911. Aug. 30. Condition: temperature 102.2° F.; legs completely, arms partially paralyzed; intercostal muscles completely, diaphragm partially paralyzed. Sept. 1. Death. Blood collected aseptically from the heart post mortem.

[6] In each case the numerator of the fraction denotes the day of the disease and the denominator the day of the paralysis on which the blood was collected from the arm vein for injection.

Sept. 2. Injected 3 c.c. of the defibrinated blood intracerebrally and 12 c.c. intraperitoneally. Monkey remained well.

Case 8.—J. L. Age, 5½ years. Onset July 12, 1912. July 20. Condition: temperature 105° F.; legs and muscles of shoulders paralyzed; facial muscles of right side weak; intercostal muscles paralyzed. Death same day. Blood collected aseptically from heart post mortem.

July 21. 30 c.c. of citrated blood were injected intraperitoneally into a *Macacus rhesus* monkey, and 0.75 c.c. intracerebrally and 30 c.c. intraperitoneally into another *Macacus rhesus*. Neither monkey developed symptoms of poliomyelitis.

Case 9.—S. N. Age, 6 years. Onset Sept. 28, 1912. Sept. 30. Condition: patient cyanotic and gasping for breath; intercostal muscles paralyzed and diaphragm weak. Increased globulin and cells in spinal fluid. Died same day. Blood collected aseptically from heart post mortem.

Case 10.—G. G. Age, 20 months. Onset gradual and indefinite. Sept. 30, 1912. Intercostals paralyzed; shoulder muscles and facial muscles on right side weak. Oct. 2. Death. Blood collected from heart post mortem.

Case 11.—M. K. Age, 2 years. Onset Sept. 28, 1912. Oct. 1. Temperature 102.4° F.; legs completely paralyzed; intercostals weak; pulse weak and irregular. Increased cells and globulin in spinal fluid. Oct. 4. Consolidation of both lungs. Temperature 104.5° F. Death on same day. Blood collected from heart post mortem.

Oct. 11. Injected 2.5 c.c. of the mixed defibrinated blood of these three fatal cases intracerebrally, and 7 c.c. intraperitoneally into a *Macacus rhesus* which showed excitability on Oct. 15. No paralysis occurred; and later a test inoculation with M A virus caused typical paralysis. Hence it was concluded that this monkey had not developed an abortive attack of experimental poliomyelitis from the injection of the blood.

Case 12.—J. C. Age, 9 years. Onset Oct. 5, 1912. Oct. 8. Condition: temperature 102.6° F.; muscles of right leg and both shoulder girdles paralyzed; intercostal muscles weak. Increased cells and globulin in spinal fluid. Oct. 9. Death. Blood collected from heart post mortem.

Oct. 9. 2 c.c. of the whole blood injected intracerebrally and 35 c.c. intraperitoneally into a *Macacus rhesus*. No effects followed.

These tests confirm those previously made by others with the blood from human cases of poliomyelitis. They do not, however, suffice to exclude altogether the possibility of the virus being present in the blood, since it is known that the original human strains are not as infectious for monkeys as the adapted strains. Another complicating factor may sometimes play a part in preventing infection from the blood. After the first week of acute illness immune bodies which have a neutralizing effect on the virus appear in the blood, and it is not improbable that by acting on the virus, originally of low infective power for monkeys, they may further diminish the chances of producing paralysis. In view of these considerations we have turned to the experimental cases of poliomyelitis in order to find a solution of the question.

EXPERIMENTAL.

In several instances in the course of our experimental study of poliomyelitis we have made inoculations of the blood of monkeys in the acute stage of paralysis into the brain or peritoneum of normal monkeys in order to ascertain whether the virus is thus demonstrable in the circulating blood. They all resulted negatively.

The experiments were now modified as follows: heavy emulsions of the spinal cord taken from a recently paralyzed animal were injected (a) intracerebrally and (b) both intracerebrally and intraspinously into monkeys from which blood was taken at intervals for inoculation intracerebrally into other monkeys. It was reasoned that the virus would be carried from the subarachnoid spaces into the veins of the membranes of the spinal cord and thence into the general blood. The protocols follow.

Experiment A.—Mar. 24, 1913. 3 c.c. of a suspension of glycerinated M A spinal cord were injected intracerebrally into a *Macacus rhesus.* Blood was taken one, six, twenty-four, and forty-eight hours after inoculation. Mar. 31. Paralysis of arms and back. Apr. 2. Etherized. With the blood taken at the intervals given other monkeys received intracerebral injections of 1 c.c. each. None developed symptoms of poliomyelitis.

Experiment B.—May 16, 1913. A *Macacus rhesus* was injected intracerebrally with 2 c.c., and intraspinally with 5 c.c. of emulsion of the spinal cord (M A) taken from a paralyzed monkey. Blood was taken one, six, and twenty-four hours after inoculation for reinoculation into other monkeys. May 24. Paralysis and prostration; etherized. With the blood withdrawn at intervals, from 2 to 3 c.c. were injected intracerebrally into *Macacus rhesus* monkeys, none of which showed symptoms of poliomyelitis.

Hence under conditions favorable for the passage of the virus into the blood none could be detected by the experiments performed. However, as will appear later, the negative results obtained might have been due in part to the relatively weak virulence of the strain of virus employed since the M A virus had at this period lost a large part of its activity.[7] When a highly active virus is employed for intracerebral inoculation it has happened exceptionally that the blood is infective when taken early in the paralysis or at the onset of symptoms,[8] as is shown in the following protocol.

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[7] Flexner, S., Clark, P. F., and Amoss, H. L., *Jour. Exper. Med.*, 1914, xix, 195.

Experiment C.—A *Macacus rhesus* had been inoculated intracerebrally with K virus on Nov. 17, 1913, and on Nov. 24 had begun to show definite early symptoms of infection. Blood was immediately withdrawn and defibrinated, and 4 c.c. were injected intracerebrally into another rhesus monkey which on Nov. 29 was somewhat excitable. On Nov. 30 the latter showed paralysis of the left leg. On Dec. 1 the arms and back were weak. On Dec. 2 animal died. Typical lesions of poliomyelitis were present.

We next resorted to the active K virus for the double inoculation.

Experiment D.—Jan. 5, 1914. A needle was introduced into the lumbar cistern of the spinal cord of a *Macacus rhesus,* and a few drops of clear cerebrospinal fluid were permitted to escape. While in position 2 c.c. of an emulsion of K virus were injected intracerebrally. The injection was followed at first by the escape of clear fluid, and in two and a half minutes turbid blood-tinted fluid escaped from the lumbar puncture needle. On completion of the intracerebral inoculation 2 c.c. of the emulsion were injected intraspinously. Five hours and seventy-two hours later blood was taken from the median basilic vein and defibrinated for intracerebral inoculation. Jan. 8. Excitable; left arm and back weak; nystagmus. Jan. 10. Paralysis of arms. Jan. 12. Death. Lesions of poliomyelitis present.

Two Macacus monkeys were injected intracerebrally, one with the defibrinated blood taken five hours, and the other three days (at onset of paralysis) after the double inoculation. Both remained well.

This experiment indicates that the detection by inoculation of even a highly pathogenic virus in the blood following combined intracerebral and intraspinous injection is difficult and uncertain. That the virus may sometimes appear in the blood after intraspinous inoculation is shown by the detection of the globoid microörganisms in the film preparations in one such instance.[9]

The problem was now approached from another side. The virus was injected into the veins and the blood tested subsequently in order to ascertain whether it remains and multiplies there or is filtered out and removed. While doubt may exist as to whether the virus enters the blood in quantity and remains there in the case of intracerebral inoculation there can be no doubt of the entrance by the intravenous mode of inoculation, in which the quantity introduced may also be varied at will. Hence a series of experiments was made by injecting the virus intravenously and withdrawing blood at intervals from a distant vein and injecting it intracerebrally into normal animals.

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[8] Zappert, J., Leiner, K., and von Wiesner, R., *loc. cit.*

[9] Amoss, H. L., *Jour. Exper. Med.,* 1914, xix, 212.

Experiment E. Macacus rhesus 1.—Oct. 30, 1913, 3 P. M. 180 c.c. of a filtrate of K virus were injected into the right external saphenous vein. 4 P. M. Sufficient blood was withdrawn from the left external saphenous vein to yield 4 c.c. after defibrination. Oct. 31, 3 P. M. The same quantity was withdrawn from the left leg, and the defibrinated blood specimens were inoculated into two *Macacus rhesus* monkeys, 2 and 3. Nov. 9. Very weak; excitable. Died during night. Lesions of poliomyelitis present in spinal cord. Control died in six days. Typical lesions.

Monkey 2.—Oct. 30. One hour blood specimen inoculated intracerebrally. Nov. 9. Excitable and weak. Nov. 10. Left facial paralysis; ataxic. Nov. 12. Prostrate. Etherized. Lesions of poliomyelitis were present.

Monkey 3.—Oct. 31. 24 hour specimen inoculated intracerebrally. Nov. 10. Excitable; back and arms paralyzed. Nov. 11. Prostrate. Etherized. Lesions of poliomyelitis were present.

Experiment F. Macacus rhesus 1.—Nov. 14, 1913, 10.50 A. M. 100 c.c. of a filtrate of K virus were injected into a vein of the right leg. 12 M. Sufficient blood was withdrawn from a vein in the left leg to yield 4 c.c. after defibrination. Nov. 15, 11 A. M. Same quantity of blood withdrawn. Nov. 16, 11 A. M. Same quantity withdrawn. Nov. 17, 11 A. M. Same quantity withdrawn. Nov. 24. Excitable; left leg paralyzed; left facial paralysis. Sufficient blood withdrawn to yield 4 c.c. when defibrinated. Nov. 25. Legs, arms, and back weak or paralyzed. Dec. 1. Died. Lesions of poliomyelitis were present.

Monkey 2.—Nov. 14. One hour blood specimen was injected intracerebrally into a *Macacus rhesus.* Nov. 18. Excitable; left facial paralysis. Nov. 19. Extremities paralyzed. Nov. 20. Prostrate. Etherized. Lesions of poliomyelitis.

Monkey 3.—Nov. 15. 24 hour specimen inoculated intracerebrally. No symptoms developed.

Monkey 4.—Nov. 16. 48 hour specimen inoculated intracerebrally. Nov. 22. Excitable. Arms paralyzed. Nov. 24. Prostrate. Died. Lesions of poliomyelitis.

Monkey 5.—Nov. 17. 72 hour specimen inoculated intracerebrally. Nov. 24. Excitable. Arms and legs paralyzed. Blood was withdrawn for reinoculation, and employed in experiment C (page 10). Etherized. Lesions of poliomyelitis were present.

Monkey 6.—Nov. 24. 4 c.c. of defibrinated blood, taken 10 days after the intravenous injection of the filtrate when paralysis appeared, was inoculated intracerebrally into monkey 6. No symptoms appeared.

These experiments show clearly that when large and overwhelming quantities of an active filtrate are injected into the circulation the virus persists in the blood for seventy-two hours at least. Finally, it appears to be removed, as ten days after its injection, at the period of the onset of paralysis, it may not be detectable by the inoculation test. The failure of the monkey inoculated with the twenty-four hour specimen in experiment F to respond may have been due to resistance in the animal rather than to absence of the virus; however, this animal when subsequently tested with an emulsion became typically paralyzed. That the ten day specimen of blood was no longer infectious is not remarkable in view of the many negative results from inoculations of blood at the beginning of the paralysis; but the successful result (experiment C, page 10) in which the blood was infectious at the onset of the paralysis seven days after an intracerebral injection shows that the active virus may pass into the blood from the nervous tissues in which it has multiplied and may survive there for a time.

The infectivity of the blood is affected by the amount of virus injected. Experiments were made also with intravenous injections of ten cubic centimeters of filtrate.

Experiment G.—Dec. 15, 1913. 10 c.c. of filtrate of active K virus were injected into the right external saphenous vein of a *Macacus rhesus.* Dec. 16 (24 hours later) and Dec. 17 (48 hours later) 5 c.c. of blood withdrawn from the left external saphenous vein were defibrinated and each lot was injected intracerebrally into a *Macacus rhesus.* None of the three monkeys of this series developed symptoms.

Experiment H. Macacus rhesus 1.—Jan. 5, 1914. 10 c.c. of filtrate of active K virus were injected intravenously. Six and twenty-four hours later 5 c.c. of blood were withdrawn, defibrinated, and injected separately into *Macacus rhesus* monkeys 2 and 3. The animal receiving the intravenous injection showed indefinite symptoms of excitability but developed no paralysis; it gradually became thinner and weaker and died on the twenty-first day after the injection. No apparent cause of death was found in the viscera. No lesions of poliomyelitis existed.

Monkey 2.—Jan 5. Received the 5 hour specimen of blood intracerebrally. Jan. 14. Excitable; arms paralyzed. Jan. 15. Legs paralyzed; prostrate. Jan. 17. Dead. Lesions of poliomyelitis present.

Monkey 3.—Jan. 6. Received the 24 hour specimen of blood intracerebrally. Jan. 14. Excitable; left arm weak. Jan. 15. Arms, legs, and back weak or paralyzed. Jan. 18. Prostrate. Jan. 24. Prostrate, moribund. Death hastened by ether. Lesions of poliomyelitis were present.

Experiments G and H bring out clearly the uncertainty of the intravenous mode of inoculation first in causing paralysis and second in maintaining the infectivity of the blood. It would appear that only when the blood is overwhelmed by the virus is it certainly infectious over a period of three days or less. Moreover, the failure of an intravenous dose of ten cubic centimeters of filtrate of K virus to cause paralysis when 0.2 of a cubic centimeter or less is a certainly effective dose by intracerebral injection, not only emphasizes the relative susceptibilities of monkeys to the two modes of inoculation but indicates also the possession of mechanisms by the body capable of excluding the virus within the blood from the nervous tissues. Whether it is the choroid plexus that is responsible or some other structure can only be surmised. It is conceivable that access of the virus to the central nervous system is secured only by way of the cerebrospinal fluid, in which case the virus within the blood must first penetrate the barrier of the choroid plexus which possibly takes place only when overwhelming doses are injected intravenously. When the virus is successfully inoculated subcutaneously or intraperitoneally it is always possible that the penetration to the central nervous organs is by way of the nerves. Hence the results may bear on the mode of infection of poliomyelitis in man.[10] In this connection it may be stated that the incubation period of the disease is longer with intravenous than with intracerebral inoculation. With the latter the average in a series of ten animals was 6.6 days and with the former in a series of six animals it was 10 days.

That the degree of virulence of the strain has a share in the effects is clearly illustrated by an experiment carried out with M A virus at a time when its infectious power had diminished.

Experiment I.—Oct. 3, 1913. 100 c.c. of filtrate of M A virus were infused into the leg vein of a *Macacus rhesus*. One hour and twenty-four hours later blood was withdrawn from the opposite leg and defibrinated. 3 c.c. were inoculated intracerebrally into each of two *Macacus rhesus* monkeys. None of the monkeys inoculated showed symptoms.

This experiment may have a bearing upon the unsuccessful inoculations with blood from human cases. There is little doubt of the presence of the virus in the one hour and the twenty-four hour samples of blood; and yet the quality of the virus was such that it failed to cause infection even when employed for intracerebral inoculation.

[10] Flexner, S., *Jour. Am. Med. Assn.*, 1910, lv, 1105.

EXPERIMENTS WITH STOMOXYS CALCITRANS.

To the data on the infectivity of the blood given in the preceding pages there may be added briefly the results of a few experiments carried out with *Stomoxys calcitrans.* Rosenau and Brues[11] reported several instances of successful transmission of experimental poliomyelitis by means of the biting stable fly, a fact that was at first quickly confirmed by Anderson and Frost.[12] Through the courtesy of Dr. Rosenau one of us (Clark) was enabled to study his method of experimentation so that in the tests made by us Rosenau's method could be followed. Highly active M A virus was used in the experiments.

Experiment 1.—Oct. 1, 1912. 2 c.c. of a suspension of the spinal cord were inoculated intracerebrally into a *Macacus rhesus.* Oct. 4. Excitable. Oct. 6 Partial paralysis. Oct. 7. Prostrate. Oct. 9. Death.

On Oct. 4 and each day thereafter until Oct. 9 about 200 *Stomoxys calcitrans* were permitted to feed on the inoculated monkeys for two or three hours daily. The stable flies were caught in stables in New York City, and the losses from death were made up by adding fresh flies from time to time. The total number of flies employed in the experiment was about 400.

Oct. 7. A *Macacus rhesus* was inoculated intracerebrally with M A virus. Paralysis occurred on Oct. 16, prostration on the 17th, and death on the 18th. The same flies that fed on the preceding monkey were allowed to feed on this animal on Oct. 12 and again on Oct. 14, 15, 16, and 17. These flies were, therefore, permitted to feed ten times upon infected monkeys within fourteen days, five of the feedings taking place before, and five after the onset of paralysis.

From Oct. 6 to 18, except on three days, the 12th, 15th, and 16th, when fresh flies were introduced into the cage and fed first on infected animals, all these flies were given access, for two to three hours at a time, to four healthy monkeys (two *Macacus rhesus* and two *Macacus cynomolgus*). The healthy monkeys were therefore exposed to the flies ten times, covering a period of thirteen days. None of the monkeys developed symptoms of poliomyelitis.

At the conclusion of the feedings the dead flies were collected, ground up, and converted into a Berkefeld filtrate which was inoculated intracerebrally into a *Macacus rhesus* with negative result.

Experiment 2.—Six *Macacus rhesus* monkeys were inoculated intracerebrally between Oct. 18 and Nov. 9, 1912, with M A virus, and a *Macacus cynomolgus* with tonsils from a fatal human case of poliomyelitis. The rhesus monkeys became paralyzed and succumbed; the cynomolgus developed a partial paralysis and was etherized.

Stomoxys were allowed to feed on at least one of the monkeys of this series twenty-one times between Oct. 22 to Nov. 17, or a period of twenty-seven days.

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[11] Rosenau, M. J., and Brues, C. T., Fifteenth International Congress of Hygiene and Demography, Washington, 1912.

[12] Anderson, J. F., and Frost, W. H., *Public Health Reports,* 1912, xxvii, 1733.

Monkeys in the preparalytic and paralytic stages were exposed to the flies. Since the death losses were large, about 1,400 flies in all were used in the experiment.

Beginning on Oct. 25 two healthy animals, one *Macacus rhesus* and one *Macacus cynomolgus,* were exposed to the bites of this lot of flies, for two to three hours at each exposure, on twenty-one days. The rhesus monkey appeared somewhat excitable on Nov. 4, and the excitability persisted until Nov. 11, when an examination of the cerebrospinal fluid showed it to be normal. No paralysis developed. The cynomolgus became somewhat excitable and developed weakness of one arm on Nov. 1. The condition did not progress, and on Nov. 4 the animal was etherized. Reinoculation of the spinal cord gave a negative result, and microscopical examination of the spinal cord and intervertebral ganglia showed them to be normal. Possibly the arm may have been hurt by the handling of the animal. This cynomolgus was replaced by another which was exposed to the flies from Nov. 4 to 17. It remained well.

At the conclusion of the experiment a filtrate was prepared from the dead flies, which was without effect when inoculated intracerebrally into a *Macacus rhesus.*

Experiment 3.—About 100 Stomoxys were allowed to feed on a *Macacus rhesus* during the two days following injection of a suspension of M A virus (spinal cord), and 150 Stomoxys were permitted to feed on a paralyzed and moribund monkey. The two lots were ground together and the filtrate from this emulsion was inoculated intracerebrally and intraperitoneally into a *Macacus rhesus.* The animal remained well.

The experiments conducted with Stomoxys gave negative results. In view of the tests on the infectivity of the blood given in the first part of this paper they are quite comprehensible, since it is only under exceptional circumstances that in intracerebrally inoculated monkeys the virus can be demonstrated in the blood by inoculation tests. The negative character of the tests with Berkefeld filtrates prepared from the bodies of the dead flies derives some significance from the single and exceptional successful inoculation with a similar filtrate prepared from *Cimex lectularius* (the bedbug), as reported by Howard and Clark.[13] Finally, it should be stated that a second series of experiments with the stable fly conducted by Anderson and Frost[14] was wholly negative, as were the comprehensive and critical experiments carried out by Sawyer and Herms.[15]

[13] Howard, C. W., and Clark, P. F., *Jour. Exper. Med.,* 1912, xvi, 850.

[14] Anderson, J. F., and Frost, W. H., *Public Health Reports,* 1913, xxviii, 833.

[15] Sawyer, W. A., and Herms, W. B., *Jour. Am. Med. Assn.,* 1913, lxi, 461.

SUMMARY.

Specimens of human blood taken during the paralytic stage of poliomyelitis and post mortem have proved not to be capable of infecting Macacus monkeys.

Specimens of monkey blood taken at various stages of experimental poliomyelitis have not proved as a rule to be capable of infecting monkeys. In a single instance, among ten tests, infection was secured with a specimen of blood removed at the beginning of the paralysis on the seventh day following an intracerebral inoculation.

When suspensions of the spinal cord from a paralyzed monkey have been injected into the brain or simultaneously into the brain and spinal canal, the blood removed from one to forty-eight hours later failed to cause paralysis after intracerebral injection.

When large volumes of active filtrate are injected into the circulation the blood remains infective for seventy-two hours at least, but may be no longer infective after ten days when the paralytic symptoms first appear. When, however, the filtrate is injected in smaller amount or when a filtrate of a less active virus is employed in large quantity, the blood either fails to convey infection or conveys it irregularly.

It is only when overwhelming quantities of an active virus are injected into the blood that paralysis results. The injection of moderate doses is not followed by paralysis, although the virus may still be detected in a blood sample twenty-four hours after the injection. The existence of a mechanism capable of excluding the virus within the blood from the central nervous organs is therefore inferred.

Infection is accomplished far less readily through the circulation than by means of the more direct lymphatic and nervous channels of communication with the central nervous system.

Several series of feeding experiments conducted with the biting stable fly (*Stomoxys calcitrans*) resulted negatively.

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A FURTHER STUDY OF NITROGEN RETENTION IN THE BLOOD IN EXPERIMENTAL ACUTE NEPHRITIS.*

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In a previous communication[1] we reported the results of the study of experimental acute nephritis in the cat produced by uranium nitrate, by potassium chromate, and by cantharidin. It was shown that these forms of nephritis result in moderate but distinctive retention in the blood of non-protein and urea nitrogen; that from the anatomical point of view the almost purely tubular forms of nephritis result in moderate retention of nitrogen; and that the additional anatomical involvement of the glomerulus leads to a somewhat greater retention. Since the publication of this report the same methods have been applied in other studies. Frothingham, Fitz, Folin, and Denis[2] have shown that the nitrogen retention and phenolsulphonephthalein excretion are parallel and in accordance with the degree of anatomical lesion in uranium nephritis of the rabbit, and that nitrogen retention lags somewhat behind the diminished excretion of the dye. Farr and Austin[3] have shown that in human cases the appearance of chronic passive congestion of the kidneys leads to no increase in the total non-protein nitrogen; that in chronic nephritis associated with marked albuminuria and edema there is little if any increase; that in chronic nephritis with hypertension

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[1] Folin, O., Karsner, H. T., and Denis, W., Nitrogen Retention in the Blood in Experimental Acute Nephritis of the Cat, *Jour. Exper. Med.*, 1912, xvi, 789.

[2] Frothingham, C., Jr., Fitz, R., Folin, O., and Denis, W., The Relation between Non-Protein Nitrogen Retention and Phenolsulphonephthalein Excretion in Experimental Uranium Nephritis, *Arch. Int. Med.*, 1913, xii, 245.

[3] Farr, C. B., and Austin, J. H., The Total Non-Protein Nitrogen of the Blood in Nephritis and Allied Conditions, *Jour. Exper. Med.*, 1913, xviii, 228.

there is a well marked increase in total non-protein nitrogen with a percentage increase of the ammonia-urea fraction; and that there is a concordance between the appearance of uremia and increase in the total non-protein nitrogen. Folin, Denis, and Seymour[4] have shown that in cases of chronic interstitial nephritis with high blood pressure it is possible by low protein diet to reduce the total non-protein nitrogen practically to normal, and they state that there is "no marked connection between the blood pressure and the degree of retention, indeed it is doubtful whether there is any connection." They also found no close connection between phenolsulphonephthalein excretion and nitrogen retention, presumably because the latter is so much under the influence of diet.

The purpose of the present communication is to present observations of other varieties of experimental acute nephritis than those presented in the first publication, thus including the forms produced by a specific hemolytic immune serum, by sodium arsenate, by diphtheria toxin, and by tartaric acid. The same technique was employed as in the preceding study, and, in addition, as often as possible two determinations of the normal blood were made in each experiment. Unsuccessful attempts were made to produce an experimental nephritis of the cat by the use of rattlesnake venom.[5] A cat weighing 2,500 grams died in thirty-six hours from a hypodermatic injection of 0.010 of a gram of dried venom; a cat weighing 2,200 grams survived an injection of 0.009 of a gram of dried venom; and a cat weighing 3,300 grams survived an injection of 0.013 of a gram of dried venom. None of the animals showed any retention of non-protein nitrogen, and histologically their kidneys showed nothing that could be interpreted as nephritis.

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[4] Folin, O., Denis, W., and Seymour, M., The Non-Protein Nitrogenous Constituents of the Blood in Chronic Vascular Nephritis (Arteriosclerosis) as Influenced by the Level of Protein Metabolism, *Arch. Int. Med.*, 1914 (in press). Seymour, M., The Effect of Nitrogenous Waste Products in the Blood in Chronic Interstitial Nephritis, *Boston Med. and Surg. Jour.*, 1913, clxix, 795.

[5] The dried venom of *Crotalus adamanteus* was given us by Dr. Joseph MacFarland of Philadelphia.

IMMUNE SERUM NEPHRITIS.

It has been pointed out by Pearce and Eisenbrey[6] that the nephritis produced by a hemolytic immune serum differs from that produced by a specific nephrotoxic serum principally in that the former shows more marked glomerular change histologically. Physiologically, however, there is no notable difference between the two, both showing the vascular reactions of a tubular nephritis. Because of the greater ease of preparation, hemolytic immune serum was used in the present study. In the cat there is, in the early stage of this variety of nephritis, moderate cloudy swelling of the tubular epithelium most noticeable in the distal convoluted tubules associated with moderate swelling of the capillary endothelium of the tuft and the appearance of much albuminous precipitate in the subcapsular space. Hyaline and granular casts are frequent in the ascending loops of Henle, but the picture throughout is obscured by the bile and hemoglobin staining of the tissues. After the disappearance of this staining the glomerular change is somewhat more marked and the degeneration of the distal convoluted tubules persists, in diminishing degree of severity, up to twelve days, the longest period of observation.

Cat 15.—Weight 1,650 gm. Given intravenously 0.8 c.c. of specific hemolytic immune serum (about 0.5 c.c. per kilo). Bled on the 2d, 3d, 4th, 8th, 10th, and 12th days. Hemoglobinuria for 24 hours. Albuminuria appeared on the 2d day and continued until the 4th day, but had disappeared on the 8th day. Killed with chloroform on the 12th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (8 dys.)	42 mg.[7]	27 mg.
Before injection (4 dys.)	41 mg.	27 mg.
21 hrs. after injection	52 mg.	35 mg.
45 hrs. after injection	47 mg.	31 mg.
69 hrs. after injection	48 mg.	23 mg.
7 dys. after injection	52 mg.	27 mg.
9 dys. after injection	42 mg.	23 mg.
11 dys. after injection	55 mg.	22 mg.

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[6] Pearce, R. M., and Eisenbrey, A. B., A Physiological Study of Experimental Nephritis Due to Bacterial Poisons and Cytotoxic Sera, *Jour. Exper. Med.*, 1911, xiv, 306.

[7] In all cases the figures represent milligrams per 100 c.c. of blood.

Cat 16.—Weight 1,950 gm. Given intravenously 1.5 c.c. of specific hemolytic immune serum (about 0.75 c.c. per kilo). Bled on the 2d day. Albuminuria and hemoglobinuria on the 2d day. Killed with chloroform on the 2d day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (3 wks.)	39 mg.	26 mg.
Before injection (2 wks.)	39 mg.	25 mg.
24 hrs. after injection	105 mg.	84 mg.

Cat 17.—Weight 2,150 gm. Given intravenously 1.6 c.c. of specific hemolytic immune serum (about 0.75 c.c. per kilo). Bled on the 2d day. Hemoglobinuria and albuminuria on the 2d day. Found dead on the morning of the 3d day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 wk.)	40 mg.	24 mg.
Before injection (4 dys.)	34 mg.	19 mg.
24 hrs. after injection	80 mg.	45 mg.

It will be seen that a relatively small dose of hemolytic immune serum is capable of producing a slight retention of nitrogen in the blood and that larger doses result in well marked retention of nitrogen with a considerable increase in the urea fraction. Thus a nephritis which shows extensive tubular change histologically and moderate glomerular change, a nephritis which physiologically is tubular in character, produces a moderate degree of nitrogen retention.

ARSENIC NEPHRITIS.

Arsenic produces a nephritis in the cat which in the earlier stages shows histologically only slight glomerular change in the form of albumin in the subcapsular space and an occasional swollen endothelial cell. The tubular epithelium shows a rapidly advancing cloudy swelling, proceeding particularly in the proximal convoluted tubules to marked necrosis; there is much granular and hyaline cast formation. In the later stages (two weeks) the epithelium shows a marked tendency to recovery and the glomerulitis becomes somewhat more distinct but never very severe. This form of nephritis in dogs is primarily vascular in the physiological sense,[8] but in its later stages (three to five days) is more nearly like the tubular forms. Examinations of the urine have shown an increased output of nitrogen. The highly fatal character of arsenic intoxication renders the study of the condition somewhat more difficult than in the case of the other nephrotoxic agents.

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[8] Pearce, R. M., Hill, M. C., and Eisenbrey, A. B., Experimental Acute Nephritis: The Vascular Reactions and the Elimination of Nitrogen, *Jour. Exper. Med.*, 1910, xii, 196.

Cat 18.—Weight 2,400 gm. Given subcutaneously 0.025 gm. of sodium arsenate (about 0.010 gm. per kilo). Bled after 6 hours and on the 2d, 5th, and 6th days. No albuminuria up to the 11th day. On the 11th day given 0.075 gm. of sodium arsenate (about 0.030 gm. per kilo). Bled after 18 hours, after showing slight albuminuria; killed with chloroform after bleeding.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 mo.)	40 mg.	20 mg.
Before injection (3 wks.)	40 mg.	21 mg.
3 hrs. after 1st injection	46 mg.	28 mg.
30 hrs. after 1st injection	47 mg.	28 mg.
78 hrs. after 1st injection	41 mg.	23 mg.
102 hrs. after 1st injection	50 mg.	30 mg.
18 hrs. after 2d injection	79 mg.	66 mg.

Cat 19.—Weight 2,500 gm. Given subcutaneously 0.075 gm. of sodium arsenate (0.030 gm. per kilo). Bled after 6 hours, at which time moderate albuminuria was found. Found dead at the end of 24 hours.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (3 dys.)	38 mg.	20 mg.
6 hrs. after injection	40 mg.	28 mg.

Cat 20.—Weight 2,080 gm. Given subcutaneously 0.045 gm. of sodium arsenate (about 0.0225 gm. per kilo). Bled on the 2d, 3d, 5th, 7th, 8th, and 14th days. Albuminuria appeared on the 2d day and continued until the 7th, but had disappeared on the 14th day. Killed with chloroform on the 18th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (19 dys.)	44 mg.	28 mg.
Before injection (2 wks.)	43 mg.	28 mg.
24 hrs. after injection	63 mg.	50 mg.
48 hrs. after injection	55 mg.	39 mg.
96 hrs. after injection	50 mg.	40 mg.
120 hrs. after injection	47 mg.	30 mg.
2 wks. after injection	39 mg.	17 mg.

Cat 21.—Weight 1,850 gm. Given subcutaneously 0.043 gm. of sodium arsenate (about 0.0225 gm. per kilo). Bled on the 2d and 4th days. Albuminuria appeared on the 2d day and continued until death. Animal was found dying on the 5th day, and blood was taken from the posterior vena cava as death occurred.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen
Before injection (6 dys.)	42 mg.	27 mg.
Before injection (5 dys.)	43 mg.	27 mg.
24 hrs. after injection	52 mg.	39 mg.
72 hrs. after injection	125 mg.	98 mg.

Cat 22.—Weight 2,000 gm. Given subcutaneously 0.050 gm. of sodium arsenate (0.025 gm. per kilo). Bled on the 2d, 4th, and 10th days. Albuminuria appeared on the 2d day and continued until death. Killed with chloroform on the 10th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (5 dys.)	39 mg.	25 mg.
Before injection (3 dys.)	45 mg.	30 mg.
24 hrs. after injection	95 mg.	68 mg.
72 hrs. after injection	40 mg.	27 mg.
216 hrs. after injection	57 mg.	29 mg.

From the results it will be seen that arsenic nephritis is accompanied by a moderate retention of nitrogen in from twenty-four to seventy-two hours, but that in the early stages, when physiologically it is of vascular type the retention is extremely slight. In the animal with most marked nitrogen retention the changes histologically were almost solely tubular, even at a stage when physiologically the condition is a tubular nephritis. From the point of view of nitrogen retention, the nephritis of arsenic shows the most marked retention at the time when the tubular changes are most prominent.

DIPHTHERIA TOXIN NEPHRITIS.

The nephritis produced by a single toxic dose of diphtheria toxin is essentially an acute glomerular nephritis.[9] There is swelling of the glomerular tuft with slight endothelial proliferation, infiltration of the polymorphonuclear leucocytes, occasionally pyknotic nuclei, albumin in the subcapsular space, occasionally hyaline thrombi, and swelling of the subcapsular epithelium. The tubular epithelium shows moderate cloudy swelling in the earlier cases with the accumulation of a moderate amount of granular material in the lumina of the tubules. Later there is more marked degeneration and small areas of actual necrosis. The change affects all the tubular epithelium, but most markedly that of the proximal convoluted tubules.

For the sake of uniformity the dose is given in units. It has been shown by Paton, Dunlop, and Macadam[10] that there is an increased output of nitrogen following the administration of diphtheria toxin, which they believe to be due to the increased catabolism of fever, but there is nothing in their report to indicate whether or not they gave sufficiently large doses to produce a distinct nephritis. Pearce and Eisenbrey[11] regard the earlier stages of the nephritis as physiologically tubular in character and the later stages as probably vascular.

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[9] The toxin used was furnished and titrated by Dr. Theobald Smith.

[10] Paton, N., Dunlop, J. C., and Macadam, I., On the Modifications of the Metabolism Produced by the Administration of Diphtheria Toxin, *Jour. Physiol.*, 1899, xxiv, 331.

Cat 23.—Weight 2,850 gm. Given 10 units of diphtheria toxin subcutaneously (about 3 units per kilo). Bled on the 2d day. Well marked albuminuria on the 2d day. Found dead on the morning of the 4th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (5 dys.)	41 mg.	25 mg.
Before injection (1 dy.)	42 mg.	28 mg.
24 hrs. after injection	50 mg.	33 mg.

Cat 24.—Weight 2,350 gm. Given 4.5 units of diphtheria toxin subcutaneously (about 2 units per kilo). Bled on the 3d and 5th days. No urine at the end of 24 hours; did not eat well after injection. Slight albuminuria at the end of 48 hours, increasing in amount until death. Found dead on the 6th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (5 dys.)	38 mg.	22 mg.
Before injection (same dy.)	38 mg.	23 mg.
48 hrs. after injection	61 mg.	39 mg.
96 hrs. after injection	227 mg.	186 mg.

Cat 25.—Weight 2,950 gm. Given 9 units of diphtheria toxin subcutaneously (about 3 units per kilo). Bled on the 3d and 4th days. Albuminuria appeared on the 3d day (urine lost on the 2d day), and was well marked until death. Found dead on the morning of the 5th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 wk.)	41 mg.	24 mg.
Before injection (3 dys.)	41 mg.	26 mg.
48 hrs. after injection	50 mg.	36 mg.
72 hrs. after injection	200 mg.	158 mg.

Cat 26.—Weight 2,700 gm. Given 3 units of diphtheria toxin subcutaneously (about 1 unit per kilo). Bled on the 3d and 5th days. Albuminuria appeared on the 3d day and was moderate until death. Found dead on the afternoon of the 5th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	34 mg.	17 mg.
48 hrs. after injection	80 mg.	45 mg.
96 hrs. after injection	254 mg.	216 mg.

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[11] Pearce, R. M., and Eisenbrey, A. B., *loc. cit.*

It will be seen that in the earlier stages, when the condition physiologically is tubular, there is only slight retention of nitrogen, but that later, when physiologically the condition is vascular the retention becomes extremely marked. This latter effect, however, probably has much to do with the increased catabolism of the intoxication, and, as Pearce and Eisenbrey point out, the vascular reactions are influenced at this stage by the profound and general effect of the toxin rendering accurate physiological conclusions impossible. From both histological and physiological studies there can be little doubt that there is severe glomerular change at this period of great retention, as well as distinct tubular change.

TARTARIC ACID NEPHRITIS.

That tartaric acid can produce a nephritis was first pointed out by Underhill,[12] confirmed and studied further by Underhill, Wells and Goldschmidt,[13] and also confirmed by Pearce and Ringer.[14]

The nephritis produced in the cat by the subcutaneous injection of tartaric acid (racemic, Merck) shows a most marked necrosis of the epithelium of the convoluted tubules, as described in dogs and rabbits by Underhill and Wells. There is cloudy swelling of the epithelium generally. There is much albuminous precipitate in the subcapsular space of the glomerulus, as described by Pearce and Ringer. In the later stages the epithelial change is only that of slight cloudy swelling of the epithelium of the proximal convoluted tubule and occasional glomerular tufts show slight swelling of the convoluted tubules. Two cases show well marked interstitial edema. Studies of the vascular reactions of this form of nephritis have not appeared.

[12] Underhill, F. P., The Influence of Sodium Tartrate upon the Elimination of Certain Urinary Constituents during Phlorhizin Diabetes, *Jour. Biol. Chem.*, 1912, xii, 115.

[13] Underhill, F. P., Wells, H. G., and Goldschmidt, S., Tartrate Nephritis, with Especial Reference to Some of the Conditions under Which It May Be Produced, *Jour. Exper. Med.*, 1913, xviii, 322; A Study of Renal Secretion during Tartrate Nephritis, *ibid.*, p. 347; A Note on the Fate of Tartrates in the Body, *ibid.*, p. 317.

[14] Pearce, R. M., and Ringer, A. I., A Study of Experimental Nephritis Caused by the Salts of Tartaric Acid, *Jour. Med. Research*, 1913, xxix, 57.

Cat 27.—Weight 1,650 gm. Given subcutaneously 0.75 gm. of tartaric acid in 6 c.c. of water, plus 4 c.c. of saturated sodium carbonate solution (about 0.45 gm. of tartaric acid per kilo). Bled on the 2d, 3d, 4th, 6th, 7th, 8th, 11th, and 13th days. Albuminuria appeared on the 2d day, but was absent on the 5th, 6th, and 7th days, reappeared on the 8th, 9th, and 10th days, and was absent on the 13th day. Killed with chloroform on the 14th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (2 dys.)	45 mg.	27 mg.
Before injection (same dy.)	42 mg.	24 mg.
22 hrs. after injection	69 mg.	45 mg.
46 hrs. after injection	36 mg.	20 mg.
71 hrs. after injection	40 mg.	22 mg.
118 hrs. after injection	57 mg.	31 mg.
142 hrs. after injection	54 mg.	30 mg.
11 dys. after injection	50 mg.	30 mg.
13 dys. after injection	52 mg.	31 mg.

Cat 28.—Weight 2,150 gm. Given subcutaneously 1 gm. of tartaric acid in 5 c.c. of water, plus 7 c.c. of saturated sodium carbonate solution (about 0.5 gm. of tartaric acid per kilo). Bled on the 2d and 3d days. Albuminuria appeared on the 2d day and continued on the 3d day. Found dead in cage on the morning of the 4th day probably as the result of heart puncture.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (2 dys.)	45 mg.	26 mg.
Before injection (same dy.)	46 mg.	27 mg.
24 hrs. after injection	154 mg.	116 mg.
45 hrs. after injection	222 mg.	181 mg.

Cat 29.—Weight 2,120 gm. Given subcutaneously 1.8 gm. of tartaric acid in 8.2 c.c. of water, plus 10 c.c. of saturated sodium carbonate solution (about 0.9 gm. of tartaric acid per kilo). Bled on the 2d, 3d, 5th, 6th, 7th, 8th, 11th, and 13th days. Albuminuria appeared on the 2d day, continued very small in amount, was barely perceptible on the 8th day, and became marked thereafter. Cat developed snuffles and was killed on the 13th day.

Time of bleeding	Non-protein nitrogen:	Urea nitrogen.
Before injection (2 dys.)	45 mg.	26 mg.
Before injection (same dy.)	44 mg.	26 mg.
24 hrs. after injection	132 mg.	100 mg.
45 hrs. after injection	94 mg.	66 mg.
96 hrs. after injection	38 mg.	19 mg.
120 hrs. after injection	37 mg.	19 mg.
144 hrs. after injection	40 mg.	25 mg.
7 dys. after injection	40 mg.	23 mg.
10 dys. after injection	50 mg.	30 mg.
12 dys. after injection	50 mg.	29 mg.

Cat 30.—Weight 1,800 gm. Given subcutaneously 1.6 gm. of tartaric acid in 8 c.c. of water, plus 8 c.c. of saturated sodium carbonate solution (0.9 gm. of

tartaric acid per kilo). Bled on the 2d, 3d, 4th, and 6th days. Albuminuria appeared on the 2d day and continued until death. Found dead in cage on the morning of the 7th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (2 dys.)	40 mg.	22 mg.
Before injection (same dy.)	41 mg.	24 mg.
24 hrs. after injection	166 mg.	120 mg.
48 hrs. after injection	229 mg.	190 mg.
73 hrs. after injection	156 mg.	119 mg.
129 hrs. after injection	130 mg.	100 mg.

Cat 31.—Weight 2,200 gm. Given subcutaneously 2 gm. of tartaric acid in 10 c.c. of water, plus 10 c.c. of saturated sodium carbonate solution (about 0.9 gm. of tartaric acid per kilo). Bled on the 2d and 3d days. Albuminuria appeared on the 2d day and on the morning of the 4th day. Complete anuria at the end of 24 hours, and on the 2d and 3d days. Found dead in cage on the morning of the 4th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	40 mg.	26 mg.
22 hrs. after injection	133 mg.	100 mg.
48 hrs. after injection	210 mg.	160 mg.

Cat 32.—Weight 2,200 gm. Given subcutaneously 2 gm. of tartaric acid in 9 c.c. of water, plus 6 c.c. of saturated sodium carbonate solution (about 1 gm. of tartaric acid per kilo). Bled on the 2d, 4th, and 6th days. Albuminuria appeared on the 2d day and was marked until death. Developed snuffles and was killed with chloroform on the 6th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	47 mg.	30 mg.
24 hrs. after injection	50 mg.	32 mg.
72 hrs. after injection	277 mg.	218 mg.
120 hrs. after injection	382 mg.	312 mg.

It can be seen that the degree of nitrogen retention in these animals with tartaric acid nephritis is well marked, that it appears to bear a direct ratio to the dose of tartaric acid, and that it is most marked when the lesion histologically is almost purely tubular.

SUMMARY.

These studies, like all studies of the kidney, are difficult of interpretation because of the impossibility of producing a pure glomerular or pure tubular nephritis. Arsenic nephritis in its early stage is physiologically a vascular nephritis, but anatomically tubular, and shows in this stage almost no retention of nitrogen, although studies of urinary nitrogen indicate an increased catabolism. On the other hand, diphtheria nephritis in the early stages is anatomically a nephritis with marked involvement of the glomerulus anatomically, but it is physiologically a tubular form, and in this early stage there is little or no nitrogen retention although studies of metabolism indicate that protein catabolism is increased by the administration of diphtheria toxin. In both cases, however, there appears to come a time when the excreting power of the kidney is exhausted and nitrogen accumulation occurs in the blood. This is much less marked in the arsenic nephritis, with less tubular change, than in diphtheria toxin nephritis with its marked tubular degeneration. This same late accumulation of blood nitrogen is seen in immune serum nephritis, where the tubular changes are persistent and relatively more severe than the glomerular change. It might well be said that this is no argument for the influence of the tubules in the excretion of waste nitrogen because of the general exhaustion of the organism as a whole and of the kidney in particular, and that no positive conclusions can be drawn is indicated by the opening sentence of this summary. Tartrate nephritis was at first considered as purely tubular; but the appearance of occasional glomerular change, as mentioned by Wells in one of his animals, and the fact that the presence of precipitated albumen in the subcapsular space, pointed out by Pearce and Ringer, probably indicates an increased permeability of the tuft capillaries, all lead to the conclusion that although the tubular change predominates, there is, possibly, slight alteration of the glomerulus. This form of nephritis shows the most marked retention of nitrogen, persisting even though the glomeruli show almost no change, tending to clear up with the progress of time and evidently also with the repair of the tubular change, and bearing a direct relation to the dose of tartaric acid and presumably with the degree of tubular change. It must be remembered, however, that the nephritis in the cases of greatest retention is a very severe form, and this again clouds the physiological interpretation of the results.

The study shows no reason for altering the conclusions of our earlier studies,[15] but from the interpretation accorded above it appears to throw more stress on tubular change as determining nitrogen retention. It confirms in addition the value of the methods used for studies of this type.

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[15] Folin, O., Karsner, H. T., and Denis, W., *loc. cit.*

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A NOTE ON NITROGEN RETENTION FOLLOWING REPEATED INJECTIONS OF NEPHROTOXIC AGENTS.*

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During the course of experiments previously reported[1] it was considered advisable to try to produce a subacute or chronic nephritis by the repeated injection of nephrotoxic agents and to observe the effects of the repeated injections on the amount of non-protein nitrogen in the blood. For this purpose uranium nitrate and diphtheria toxin were selected, and the technique detailed in the first study was followed. The experiments although not extensive are considered of sufficient interest to justify publication. An animal in which spontaneous chronic nephritis had occurred is included in the report because of the relation of this process to the experimental conditions studied and because of the close relation it bears to the work of Folin, Denis, and Seymour,[2] which shows that in human cases of chronic interstitial nephritis with hypertension it is possible to reduce the blood nitrogen practically to normal by the use of a low protein diet.

URANIUM NITRATE.

Cat 33.—Weight 2,670 gm. Given 0.00025 gm. of uranium nitrate daily (about 0.0001 gm. per kilo) for 5 consecutive days; then given double this dose for 12 consecutive days; then triple the primary dose for 5 consecutive days; and then quadruple the primary dose for 7 consecutive days. The cat lived for 11 days more and was found dead in the cage on the morning of the 41st day. It was observed for 41 days and received a total of 0.018 gm. of uranium nitrate, 9 times the dose necessary to produce a marked nephritis in a 4,000 gm. cat. Albuminuria appeared to a slight degree on the 3d day, becoming marked 24 hours after doubling the dose, and continuing so until the dose was discontinued, when it became slight and remained so until death.

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[1] Folin, O., Karsner, H. T., and Denis, W., Nitrogen Retention in the Blood in Experimental Acute Nephritis of the Cat, *Jour. Exper. Med.*, 1912, xvi, 789. Karsner, H. T., and Denis, W., A Further Study of Nitrogen Retention in the Blood in Experimental Acute Nephritis, *idem*, 1914, xix, 259.

[2] Folin, O., Denis, W., and Seymour, M., The Non-Protein Nitrogenous Constituents of the Blood in Chronic Vascular Nephritis (Arteriosclerosis) as Influenced by the Level of Protein Metabolism, *Arch. Int. Med.*, 1914 (in press). Seymour, M., The Effect of Nitrogenous Waste Products in the Blood in Chronic Interstitial Nephritis, *Boston Med. and Surg. Jour.*, 1913, clxix, 795.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	43 mg.	28 mg.
3d dy. of experiment	43 mg.	27 mg.
5th dy.	40 mg.	25 mg.
Dose doubled		
7th dy.	40 mg.	25 mg.
.9th dy.	40 mg.	26 mg.
11th dy.	40 mg.	25 mg.
14th dy.	41 mg.	25 mg.
16th dy.	41 mg.	22 mg.
18th dy.	43 mg.	28 mg.
Triple dose (18th dy.)		
23d dy.	53 mg.	36 mg.
Quadruple dose (23d dy.)		
25th dy.	54 mg.	39 mg.
28th dy.	91 mg.	67 mg.
30th dy.	126 mg.	113 mg.
Dose discontinued		
32d dy.	160 mg.	125 mg.
35th dy.	174 mg.	142 mg.
39th dy.	228 mg.	190 mg.
Found dead on the morning of the 41st day.		

At autopsy there was present a slight acute valvulitis (mitral), probably the mechanical effect of the frequent heart punctures, a chronic adhesive pericarditis from the same cause, fat necrosis in the abdominal fat, but an apparently normal pancreas. The kidneys showed no change except that the glomeruli were white and prominent. Histologically the interstitial tissue shows between the tubules of the cortex numerous small areas of recent overgrowth, the nuclei being large, prominent, and vesicular. The glomeruli show a normal capsule and normal capsular epithelium. The subcapsular space is filled with a hyaline albuminous precipitate. The vascular tuft occupies about the normal space in the glomerulus but is almost devoid of blood; the endothelium shows extremely large frequent and vesicular nuclei, occasional mitotic figures, occasional lymphocytes and leucocytes and a well marked increase of collagenous intercellular material, apparently representing thickened capillary walls. Occasional proximal convoluted tubules are normal, but many more show hyaline casts and granular detritus within the lumen; in these tubules the epithelium does not project the normal distance above the basement, is narrowed, but shows no necrosis. The other tubules, particularly the loops of Henle and the distal convoluted tubules, show an unusually large number of nuclei, in some cases there being two and even three nuclei in what appears to be a single cell body, although very few mitotic figures could be found. The blood vessels appear to be normal. The condition is interpreted as a subacute diffuse nephritis.

This animal had in the beginning functionally normal kidneys, which were also probably anatomically normal, for at the end of the experiment they showed neither grossly nor histologically the features of a spontaneous chronic nephritis. The repeated doses of uranium nitrate resulted in the appearance on the third day of albuminuria, which in this case can safely be interpreted as indicating functionally at least the presence of a nephritis. Not until after fifteen days had elapsed, however, was any demonstrable nitrogen retention observed and then after four daily administrations of triple the primary dose or 0.0003 of a gram of uranium nitrate per kilo, a single dose of 0.0005 of a gram being sufficient to produce a well marked nephritis in the cat.[3] This moderate retention continued until five days after the primary daily dose was quadrupled. For nine days after the drug was discontinued the nitrogen retention increased, a period during which the nephritis functionally became more marked, but in which the anatomical condition of the kidney, at least in so far as the epithelium was concerned, showed attempts at repair. It is hardly probable that the drug was retained in the body during the twelve days following the cessation of administration and hence the nitrogen retention and the final death of the animal must be looked upon as the effects of the artificially produced nephritis, even though the course of the experiment appeared to show nitrogen retention in a general way proportional to the dose of drug.

DIPHTHERIA TOXIN.

Three animals were given diphtheria toxin[4] in daily doses of 0.1, 0.25, and 0.5 of a unit per kilo respectively.

Cat 34.—Weight 3,050 gm. Given subcutaneously 0.3 of a unit of diphtheria toxin (about 0.1 of a unit per kilo) daily for 30 days with the exception of the 6th, 13th, and 26th days, making a total of 27 injections, or 8.1 units of toxin. Albuminuria appeared on the 7th day, after the administration of a total of 1.5 units of toxin, became marked on the 13th day, after the animal had received a total of 3.3 units of toxin, and continued well marked until it was killed by chloroform.

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[3] Folin, Karsner, and Denis, *loc. cit.*, p. 790 (cat 3).

[4] The toxin was furnished us and titrated by Dr. Theobald Smith.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	35 mg.	16 mg.
4th dy. of experiment	37 mg.	20 mg.
7th dy.	37 mg.	20 mg.
9th dy.	35 mg.	19 mg.
11th dy.	36 mg.	19 mg.
14th dy.	50 mg.	28 mg.
18th dy.	40 mg.	24 mg.
25th dy.	46 mg.	28 mg.
31st dy.	41 mg.	28 mg.

The autopsy showed nothing notable grossly other than the kidneys which were small, firm, and showed slight depressions in the outer surface along the lines of the larger veins; the cut surface showed almost no striations and the glomeruli were prominent and red. Histologically there is found no overgrowth of connective tissue about the surface veins or in any other part of the kidney. The glomeruli show well marked swelling of the capsular epithelium, and in many cases slight proliferation of the cells, but in no case are there found the crescentic figures of chronic capsular disease. The vascular tufts are filled with blood and although an occasional migrating leucocyte is found the tufts are practically normal. The subcapsular space contains a granular albuminous precipitate. The proximal convoluted tubules are normal except for occasional small areas of necrosis of the epithelium. The distal convoluted tubules show much formation of hyaline droplets in the epithelial cells. The tubules otherwise show finely granular albuminous material in the lumina and the larger collecting tubules show large numbers of hyaline casts. The blood vessels show nothing other than congestion. The condition is a subacute epithelial nephritis with a subacute capsular glomerulitis.

Cat 35.—Weight 2,850 gm. Given subcutaneously 0.7 of a unit of diphtheria toxin (about 0.25 of a unit per kilo) daily for 7 days. Bled on the 3d, 5th, and 8th days. Died on the 8th day as the result of heart puncture. Albuminuria appeared on the 4th day and continued in considerable quantity until death.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (1 mo.)	41 mg.	24 mg.
Before injection (3 wks.)	41 mg.	23 mg.
In the interval the cat lost weight and was ill, but recovered about 1 wk. before continuation of the experiment.		
Before injection (same dy.)	37 mg.	23 mg.
48 hrs. after beginning of injection	37 mg.	23 mg.
96 hrs. after beginning of injection	37 mg.	18 mg.
168 hrs. after beginning of injection	180 mg.	144 mg.

The autopsy performed immediately after death showed nothing notable grossly. The kidneys show histologically a well marked acute change. Throughout the organ there is a distinct perivascular infiltration of leucocytes, lymphocytes, and endothelial cells. The glomeruli show swelling, desquamation of the capsular epithelium, and occasionally pyknosis and karyorrhexis. The subcapsular space contains granular albuminous material and desquamated cells. The capillary tufts although containing blood show endothelial swelling, an occasional mitotic figure, karyorrhexis, and many leucocytes both within the capillaries and infiltrating into the tuft. The tubules show slight albuminous degeneration, particularly in the distal convoluted tubules, and the collecting tubules show a few hyaline casts. The blood vessels are normal, except that some of the smaller ones contain an excess of leucocytes. The condition is an acute diffuse nephritis with the most marked change in the glomerulus.

Cat 36.—Weight 2,270 gm. Given subcutaneously 2.5 units of diphtheria toxin (about 0.5 of a unit per kilo) daily for 5 days. Bled on the 3d and 5th days. Albuminuria on the 3d day, continuing until death. Found dead in cage on the 6th day.

Time of bleeding.	Non-protein nitrogen.	Urea nitrogen.
Before injection (same dy.)	50 mg.	38 mg.
48 hrs after first injection	40 mg.	26 mg.
93 hrs. after first injection	222 mg.	182 mg.

The autopsy showed nothing notable grossly. Histologically the kidney differs from that of cat 35 only in that there is much less infiltration of leucocytes in the glomerular tuft and in the perivascular tissue of the organ.

In cats 35 and 36 the repetition of doses produced a greater degree of retention than the single dose would have produced, but the notable retention did not appear before the third day, as was also the case with the cats reported previously, which had been given a single larger dose of toxin. These two cats had a severe acute nephritis. Cat 34 with smaller doses developed albuminuria a full week before there was any nitrogen retention; the retention was then only slight and returned to normal in four days, even though there was a continued albuminuria, and the autopsy showed a nephritis of considerable duration. Anatomically this nephritis with its slight retention differed little in extent of involvement from that seen in the other two cats, although the functional powers were widely different. The length of time occupied in the experiment with cat 34 was more than sufficient for the development of immunity; hence the metabolic effects of the later doses would probably have little effect on protein catabolism and thus give less reason for calling on the kidney to excrete large amounts of nitrogenous waste. It would seem probable, therefore, that in acute diphtheria nephritis the large final accumulation of nitrogen is dependent not only on the presence of the nephritis but to a large degree upon the increased protein catabolism.[5]

SPONTANEOUS NEPHRITIS.

Cat 37.—Weight 3,500 gm. On first examination and throughout the experiment the animal showed distinct albuminuria, but there were no casts in the urine. A diet of 80 gm. of chopped beef maintained a fairly constant weight. Since albuminuria was constant it will not be mentioned in the following notes.

Date.	Weight.	Diet.	Blood. Non-protein nitrogen.	Blood. Urea nitrogen.
Apr. 15	3,500 gm.	80 gm. meat	—	—
Apr. 17		80 gm. meat	69	35
Apr. 25		80 gm. meat	58	32
Apr. 26	3,400 gm.	160 gm. meat	—	—
Apr. 27		160 gm. meat	63	38
Apr. 29		160 gm. meat	66	50
May 1	3,600 gm.	160 gm. meat	67	50
May 3		50 gm. rice, 25 c.c. 15 per cent. cream	—	—
May 7		Same diet	54	40
May 14		(Diarrhea)	—	—
May 15		80 gm. meat; condition cleared up	—	—
May 21	3,200 gm.	80 gm. meat	72	56
May 23		80 gm. meat, 1 gm. sodium chloride	—	
May 31		80 gm. meat, 1 gm. sodium chloride	54	40
June 1		80 gm. meat	—	—
June 22	Experiment terminated. Animal killed with chloroform.			

At autopsy the heart was enlarged and had a distinctly thickened endocardium. The aorta was dilated in the ascending part. The liver showed a fatty deposit in the centers of the lobules. The kidneys were normal on the outer surface, but on the cut surface showed radial streaks of connective tissue in the cortex, especially prominent near the pyramid.

Histologically the gross findings in the heart and liver are confirmed. The kidney shows a slight overgrowth of connective tissue especially about the glomerular capsules and the blood vessels; associated in many places is moderate infiltration of lymphocytes. The glomerular capsules are slightly thickened and the capsular epithelium is normal. The subcapsular spaces contain a granular albuminous precipitate. The tufts are enlarged and show a distinct increase in the number of nuclei; there is almost no blood in the tufts and the collagenous material is increased in amount, and often hyaline. The tubules are normal except that there is a moderate amount of albuminous precipitate in the lumina, the distal convoluted tubules show thinning and increased granulation of the epithelium, and occasional hyaline casts are found in the collecting tubules. The condition is a slight chronic diffuse nephritis with slightly greater quantitative involvement of the glomerular tuft than of other parts of the organ.

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[5] Paton, N., Dunlop, J. C., and Macadam, I., On the Modifications of the Metabolism Produced by the Administration of Diphtheria Toxin, *Jour. Physiol.*, 1899, xxiv, 331.

This animal as it first came to the laboratory presented in notable degree the features of a spontaneous nephritis. Under the favorable conditions of the laboratory the degree of nitrogen retention sank considerably during the course of one week, and then with several days of excessive protein diet rose slightly, especially in the urea fraction. Four days of extremely low protein diet resulted in a decrease of the total non-protein nitrogen, but still with a relatively high urea fraction. The diet had to be discontinued because a diarrhea developed. Quite unexpectedly, a week of normal diet resulted in an excessive accumulation of nitrogen, and then the addition of sodium chloride in small quantities was followed by a decrease in retained nitrogen. This study is suggestive in connection with the studies of Folin, Denis, and Seymour,[6] but it is not conclusive. The important facts demonstrated are that a mild chronic nephritis in the cat may be accompanied by a moderate retention of nitrogen, that the degree of retention is variable, and that moderately low and extremely low protein diets may be followed by a reduction in the non-protein nitrogen of the blood.

SUMMARY.

It is possible by means of repeated injections of uranium nitrate to produce in the cat a subacute or chronic nephritis which can progress to a fatal termination and show in its course increasing accumulation of non-protein nitrogen in the blood. This nephritis differs only slightly from the spontaneous chronic nephritis of the species. Repeated doses of diphtheria toxin produce a subacute form of nephritis with only temporary or slight retention, but nevertheless anatomically a well defined nephritis. The spontaneous nephritis studied resembles more closely that produced by uranium nitrate than that produced by diphtheria toxin both histologically and from the standpoint of blood analysis.

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[6] Folin, Denis, and Seymour, *loc. cit.*

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AN EXPERIMENTAL STUDY OF THE HISTOGENESIS OF THE MILIARY TUBERCLE IN VITALLY STAINED RABBITS.*

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Plates 36 to 39.

INTRODUCTION.

The inadequacy of the methods hitherto applied to the study of the histogenesis of the tubercle led us to produce tuberculosis experimentally in vitally stained rabbits. In rabbits injected with trypan blue the Kupffer cells stain deeply, while the blood cells are free from stain, and we thought that this might throw some light on the part played by the Kupffer cells in the formation of the miliary tubercle in the liver.

HISTORICAL.[1]

Opinion is still divided as to the origin and structure of the miliary tubercle. About fifty years have elapsed since Virchow first demonstrated the tubercle, named it, and showed that it was the distinctive product of tuberculosis. Although Virchow saw little in its morphology save round cells, it was not long before Langhans found the giant cell and noted its practically constant occurrence. Schueppel discriminated the three cell types (lymphocytes, epithelioid cells, and giant cells) which we recognize to-day as being usually present, if not of invariable occurrence, and of valuable diagnostic aid. The typical arrangement or succession of these three cell types has come to be a law (Ziegler).

Though we are now aware that they are by no means pathonomic for the tubercle, nevertheless the giant and epithelioid cells are peculiar structures, identical with none of the familiar fixed cells of the body, although descended from them. Their manner of descent and transformation have consequently claimed

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[1] The literature has been reviewed by Wechsberg, Dürck, Dürck and Oberdorfer, and others, and only the more important views will therefore be given.

much interest. After the discovery by Koch in 1884 of the etiological agent of tuberculosis, many investigators began to study the formation of the tubercle after the injection of pure cultures. One view identified with the name of Metchnikoff,[2] the other with that of Baumgarten, might be said to represent the views of the French and German schools respectively. Metchnikoff maintains that the tubercle is of leucocytic origin, and Baumgarten believes that the fixed tissue cells alone are responsible for its formation.

Metchnikoff's contention must be regarded as the natural outcome of his theory of the cells concerned everywhere in the defense of the body against bacterial disease. To the chief cells he has applied the term macrophage, meaning thereby a class of large mononuclear cells doubtless identified by others as wandering cells, which Metchnikoff and his followers considered to have arisen from the overgrowth of true lymphocytes. Stages in this transformation were described, so that there was no difficulty in imagining that with the increase in cell body, the deeply-staining lymphocytic nucleus became the large clear nucleus, poor in chromatin, of the epithelioid or giant cell. The phagocytic properties of the latter, however, were the chief cause of Metchnikoff's derivation of them from his macrophage, and he emphasized the almost invariable existence of bacilli in these cells. Unquestionably the convictions of Metchnikoff came more as a general result of his notions of the cells concerned in immunity than as the result of an exhaustive morphological research, but it is interesting to find that Koch himself was disposed to regard wandering cells as the chief elements in the tubercle, even though he imagined that their subsequent death might result in the participation of various fixed cells.

Baumgarten, and many investigators after him, have established the fact that the tubercle is not merely a heaping up of preformed elements, but that active proliferative processes are always concerned in its formation. The evidence for this was clear, for frequent mitoses are found, and inasmuch as these affect the chief cells of the immediate neighborhood, regardless of where the tubercle lies, Baumgarten and others have concluded that these cells alone are essential in tubercle formation, and that the most various tissue elements, including those as far apart as connective tissue and epithelium, could play this part, but that leucocytes could not. Indeed Baumgarten denied any but the latest and most trivial participation of leucocytes in the actual beginning of the tubercle, though subsequent investigators among his own countrymen could not, of course, confirm this. The preliminary outpouring of polymorphous cells and secondary lymphocytic infiltration which the French school had so clearly seen were undeniable, though Baumgarten's adherents denied a wider interpretation of this phenomenon as completely as the French school had denied the interpretation put on mitoses, the presence of which they also admitted. According to Baumgarten's theory, Kostenitsch and Wolkow describe the inception of a tubercle as follows: (1) the formation of a serofibrinous exudate, (2) migration of polynuclear leucocytes which rapidly disintegrate, (3) proliferation of fixed tissue cells to form epithelioid cells, (4) migration of mononuclear leucocytes which take a peripheral position around the epithelioid cells, and (5) secondary migration of polynuclear elements when the tubercle degenerates.

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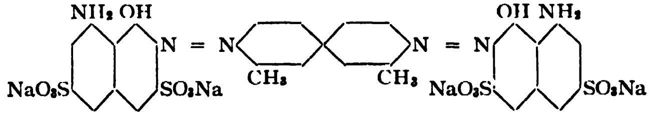
[2] Metchnikoff's views are upheld by Yersin, Borrel, Leray, and Wallgren; Baumgarten's by Klebs, Kockel, Kostenitsch and Wolkow, Wechsberg, Miller, Watanabe, Oppenheimer, Straus, Morel, and others.

The question of the origin of the cells forming the tubercle depends on our ability to distinguish sharply cell types. Few reliable criteria have been advanced for such a discrimination, although the recent work of Wallgren and Oppenheimer should be mentioned. These investigators have taken advantage with us of the fact that the production of tubercles within the liver lobule permits us to work in a relatively simple territory, where besides the blood cells and endothelium of the hepatic sinuses, only the liver cells occur, all the connective tissue elements, for instance, being absent.

Oppenheimer, starting from the fact that silver particles are phagocytized largely by Kupffer cells, has produced hepatic tubercles after collargol injections. The tubercles which appeared after the transitory leucocytosis were composed of silver-containing epithelioid and giant cells, and Oppenheimer naturally derived these from the endothelium. It is unfortunate that the conclusiveness of his experiments is open to doubt on the grounds that normally some of the blood cells engulf the silver particles. Furthermore, Wallgren, also working with rabbit liver, has reached an opposite conclusion, and substantiates fully the lymphocytic origin of the cells. There appears to be sufficient reason, therefore, for a reinvestigation of the relatively simple liver tubercle with a more decisive method of study.

MATERIAL AND METHODS.

In our experiments rabbits were used exclusively. The animals were vitally stained by repeated intravenous injection of a freshly prepared aqueous solution of trypan blue. The dye is a benzidin of the following formula,



and has been used as a vital stain, by Bouffard, Goldmann, Schulemann, Evans, and others. The staining in the experiments to be recorded may be divided into two groups: the acute staining, where daily intravenous injections of twenty cubic centimeters of 1 per cent. solution of the dye were given during a few days, and which resulted chiefly in a pigmentation of the Kupffer cells; and the chronic staining where the injections were made at longer intervals and over a longer period of time. In these animals, besides the staining of the Kupffer cells, the blood of the liver capillaries contains many vitally stained macrophages. Both types of staining are adaptable to the study of the histogenesis of the tubercle, and the method of staining will be briefly given in each experiment.

Emulsions of bovine tubercle bacilli were made in the usual manner. The bacteria were carefully removed from the glycerin bouillon medium, dried on sterile filter paper, ground, and taken up in normal salt solution. This was then put into a shaking machine for two hours. The strength of the suspension was graded so that each cubic centimeter contained one milligram of dried tubercle bacilli.

The animals were anesthetized, a loop of bowel was exposed aseptically, and ten cubic centimeters of the suspension of tubercle bacilli were injected into a small radical of the mesenteric vein. The animals were killed at varying intervals, from one half hour to eleven days, and immediately injected through the ascending aorta, by pressure, with a 10 per cent. solution of formalin. Under these conditions the tissues are rapidly fixed throughout, the intra-acinar capillaries are dilated, and most of their blood content is washed out. The liver was then removed, cut into small strips a few millimeters in thickness, and replaced in formalin. Satisfactory results were obtained from frozen sections, and these were used throughout the study. With the above fixation, sections five micra in thickness were readily obtained. The sections were stained with aqueous cochineal, which contrasted well with the blue vital stain. Other sections were stained in Ziehl-Nielson carbol fuchsin for five minutes, decolorized in 10 per cent. hydrochloric acid alcohol, thoroughly washed in distilled water, and counterstained with Delafield's hematoxylin. Weigert's fibrin stain was used in some of the early stages.

THE RESULT OF THE INJECTION OF VITAL STAIN UPON THE LIVER OF NORMAL ANIMALS.

The literature on the histology of the normal rabbit's liver has been reviewed recently by Wallgren. Concerning the Kupffer cells, Wallgren quotes Schilling who considers them to be endothelial phagocytes and functional stages of liver endothelium. He believes that they arise from normal liver endothelium, by swelling and paling of their nuclei, and that probably every endothelial cell passes through this change from time to time.

In order to check Schilling's results, we killed an animal and injected formalin through the aorta. The liver cells occasionally contain two nuclei, and a mitosis may occasionally be found. The nuclei of the endothelial cells of the capillaries are relatively wide apart, and two are found opposite each other only in rare cases. They are closely approximated to the columns of liver cells, and vary considerably in appearance. Many of them are flat, like ordinary endothelium, and there are transition forms to the larger ones with vesicular nuclei that bulge into the lumen of the capillary. The larger ones rarely contain an ingested polynuclear leucocyte or a granule of brown pigment.

EXPERIMENTAL PART.

VITALLY STAINED NORMAL ANIMALS.

Rabbit 1.—Mar. 14, 1912, 9 A. M. Injected with 20 c.c. of a 1 per cent. aqueous solution of dye in ear vein.

Mar. 15, 9 A. M. Killed. Fixed with formalin through aorta.

Liver.—The liver cells contain a few granules of trypan blue. The Kupffer cells are about normal in size and occurrence. Most of them contain a varying number of small blue granules. Endothelial cells with two nuclei or cells in mitosis are not found. Phagocytosis of polynuclear leucocytes or red blood cells is uncommon.

Blood.—The usual white blood cells, including lymphocytes and mononuclear and polynuclear leucocytes, occur and are unstained. Besides these large cells with clear non-granular protoplasm, vesicular nuclei and definite nucleoli occur. The nucleus may be round, oval, or bent. The nature of the cells is not always determinable. They appear to be similar to the large lymphocyte or polyblast emphasized by Wallgren.

Interstitial Tissue.—Normal; no vitally stained cells.

Rabbit 97 A.—Mar. 10 and 11. Intravenous injection of 20 c.c. of 1 per cent. solution of dye. Killed at the end of forty-eight hours.

Liver.—The cells contain a few small granules of vital stain. The Kupffer cells are more numerous, and many of them are increased in size. The majority contain blue granules of irregular sizes. These may be dense, or there may be only a few granules scattered near the nucleus, or even towards the tip of one of the protoplasmic processes of the cell. The dye is more abundant in the larger cells though the flat ones may contain a few granules. As the cell becomes larger, the nucleus becomes more vesicular, and rarely two nuclei may be found within one cell body. One mitotic figure was found. Phagocytosis occurs as usual.

Blood.—There are no vitally stained cells in the capillaries. The blood picture is similar to that of rabbit 1.

Interstitial Tissue.—An occasional clump of blue granules is found in the periportal tissue; but whether these are contained within the endothelium of the vessels or in the wandering cells cannot be determined.

Rabbit 200.—April 13, 14, 15, and 16. Intravenous injection of 20 c.c. of 1 per cent. solution of dye. Killed at the end of the fourth day.

Liver.—The cells contain definite, large, bright blue granules of dye, and may have two nuclei. The Kupffer cells are conspicuous. They are definitely increased in numbers and most of them are large and heavily laden with varying sized granules of dye. Some of the enlarged Kupffer cells are no longer firmly attached to the liver columns, and may be free in the lumen with their processes extending to the column of the liver cells. These cells may be cut in such a way as to appear round and free in the capillary. Not only are the Kupffer cells enlarged, but an occasional one with two or three nuclei and a rare mitotic figure may occur. In the latter the pigment granules are never found in the spindle. The nuclei of the large Kupffer cells may be irregular, and assume a polymorphous shape. The phagocytic property of the large Kupffer cells is demonstrated by the frequent finding of polymorphonuclear leucocytes or red blood cells within their bodies.

Blood.—The injection of formalin through the aorta was unsuccessful, and as a consequence, considerable blood is contained within the vessels. The larger veins contain normal unpigmented blood elements. The larger polyblast-like cells occasionally contain a few very fine granules of dye.

Interstitial Tissue.—There is an occasional pigmented cell, the nature of which could not be definitely determined.

Rabbit 80.—Six successive doses of 20 c.c. of 1 per cent. solution of dye were given on six successive days. The animal died shortly after the last injection, *i. e.*, early on the sixth day. The findings were similar to those in rabbit 200, except that two large pigmented giant cells were found in the liver.

Rabbit 49.—Nov. 23, 27, Dec. 6, 31, Jan. 5 and 13. 103 c.c. of 1 per cent. aqueous solution of trypan blue were given in approximately 20 c.c. doses. Jan. 19. Animal killed by a blow on the head, and 500 c.c. of 10 per cent. formalin injected through the aorta at a pressure of 80 mm. of mercury.

Liver.—The cells show a few granules of dye. Cells with two nuclei and occasional mitoses in the liver cells may be found. The Kupffer cells are conspicuous on account of their increased size and number, and their content of pigment granules. Kupffer cells with two or more nuclei are common and many large giant cells with innumerable nuclei are found. Frequently they occupy the position of the normal Kupffer cells, but sometimes they appear to be free. Mitosis is never demonstrable in them, but all transitions occur from those with two, three, and even more nuclei. The nuclei of the cells are large and twisted, so that they have a polymorphous appearance, and not infrequently the lobes may be connected by delicate threads of nuclear material. Phagocytosis is seen as usual.

Blood.—Besides the elements found in the previous cases, occasional large macrophages occur, often so densely pigmented that the nature of the cell is entirely obscured.

Interstitial Tissue.—The same as in the previous cases.

From the foregoing experiments it is evident that following successive intravenous injections of trypan blue the Kupffer cells become laden with the dye, enlarge, and increase in number, and finally form large free syncytial masses or giant cells containing one or several nuclei. The endothelial cells which undergo this transformation and finally become large free macrophages are filled with increasing numbers of the vital blue granules in their cytoplasm. The blood cells, on the contrary, remain free from the dye and undergo no noticeable changes. It is important to note that whereas the dye thus stimulates the formation of free endothelial cells, it does so only after an interval of four or five days; within this interval only mild proliferative changes occur, resulting merely in the enlargement of Kupffer cells and their multiplication *in situ* in the vessel wall. We shall find in tuberculosis a far more rapidly changing picture.

EXPERIMENTAL TUBERCULOSIS IN UNSTAINED RABBITS.

Rabbit 101.—Killed forty-eight hours after inoculation with a suspension of 10 mg. of bovine tubercle bacilli in superior mesenteric vein. Dense cellular masses are scattered through the liver, either in the region of the periportal vessels or in the intra-acinar capillaries.

Liver.—The cells are coarsely granular and vacuolated, but otherwise present nothing of note. The Kupffer cells are more numerous than in the normal liver, and vary in size. Many of them are much larger, and as they increase in size the nuclei become more vesicular. The smaller, more slender Kupffer cells lie against the column of the liver cells like an endothelial membrane, while the larger ones seem to be less firmly attached and may be seen almost free in the lumina of the capillaries. Here their contour is easily made out. They are spindle- or winged-shaped, with long processes that often cross the bed of the capillary before they approximate themselves to the wall of the liver columns. When cut at different angles they often resemble polyblasts. They may contain polymorphonuclear leucocytes, which in many instances are well preserved and appear to be as healthy as those free in the capillaries. Around these ingested cells is a clear vacuolar space, which may cause an indentation in the nucleus where the latter comes in contact with the ingested cell. The ingested cell gradually undergoes disintegration. The chromatin may first disappear and leave a pale homogeneously staining protoplasm containing only a few chromatin specks, or the protoplasm may be digested or shrunken, leaving a small structureless mass of chromatin. Mitotic figures occur abundantly in the Kupffer cells, and Kupffer cells with two or three nuclei are found.

Blood.—The capillaries are dilated. Here and there in the smaller capillaries a few red blood cells or a polynuclear cell may be seen. Mononuclear cells also occur, some of which are small lymphocytes. By far the greater number, however, are irregular round or oval cells whose nuclei present no constant picture. They are apparently quite active, and while some have the chromatin arranged wheel-like, others are in division. Some of the cells are definitely Kupffer cells, since they are still attached by one or more processes to the columns of liver cells. Others are not in the capillaries, but occur as small nests of cells in indentations between the liver cell and the endothelial lining. In one such area the Kupffer cell may be seen sending a process down between the liver cells. The larger clumps or plugs are in the periportal vessels, whose endothelium is swollen and partly desquamated. The lumen contains, besides these desquamated cells, one or more giant cells, polynuclear leucocytes, and mononuclear cells. One nodule, for instance, has a large, crescêntic, multinucleated giant cell at one pole. The nuclei of this cell form a horseshoe, and several small beaded bacteria in the protoplasm occur. The remainder of the nodule is composed of numerous stellate spindle and oval cells with pale vesicular nuclei. Two are dividing. The vessel wall is not clearly distinguishable, and the cellular accumulation is present to a slight extent in the surrounding tissue. The giant cells, Kupffer cells, and spindle or oval cells contain the bacteria, which occur occasionally in polynuclear leucocytes also.

In some areas pictures occur which suggest the possibility that these giant cells may increase in size by fusion. Cells with nucleus and protoplasm similar to that of the giant cell are found in close apposition to each other.

Interstitial Tissue.—In the region of the periportal spaces the interstitial tissue is obscured on account of the frequent localization of the clumps in these areas, described above. Fibrin could not be demonstrated in the clumps.

Rabbit 84.—Killed seven days after inoculation.

Liver.—The section shows the liver tissue to be infiltrated with large nodules which tend to become confluent. The cells show nothing of note except an atrophy where the columns are pressed apart by the intravascular nodules. Kupffer cells are numerous. Many of them are large and multinucleated. Mitosis is frequent and was found in one cell with three nuclei. Cells may be seen dividing with the line of division perpendicular to the long axis of the vessel. Some of the Kupffer cells are more or less rounded and indistinguishable from the polyblasts in the capillaries except by their processes.

Blood.—The cell picture resembles that found in the previous experiment. Polyblast-like cells are abundant and many show mitosis. They are also found in nests beneath the endothelium.

Plugs.—The smaller plugs have a central giant cell, surrounded by Kupffer cells and large round cells. The larger nodules show central caseation with a peripheral zone composed of the above cell types.

Bacteria are abundant and occur exclusively within the bodies of cells. The giant cells and particularly the Kupffer cells contain the organisms.

Summary.—The bacteria at the end of forty-eight hours are all intracellular, and frequent degenerative forms are encountered. The polynuclear leucocytes rarely contain tubercle bacilli, while the giant cells, Kupffer cells, and mononuclear cells contain them in the order named.

The Kupffer cells apparently are active. Frequent mitoses are found in them and Kupffer cells with two or more nuclei occur. These may form giant cells which may either occupy their normal position or lie free in the lumen of the capillary. The giant cells do not show mitoses, but their nuclei are tortuous and polymorphous suggesting multiplication by budding. Elsewhere, within the center of tubercles pictures are occasionally encountered which suggest the possible formation of giant cells, or rather their increase in size by fusion, but these pictures are rare and not convincing.

Besides polynuclear leucocytes and definite lymphocytes in the capillaries, other large white cells occur which are difficult to classify. These cells are round or oval with homogeneously staining protoplasm and varying types of nuclei. They frequently show division. They are probably the cells spoken of as polyblasts by Wallgren. Some of them have streamers that extend to a neighboring Kupffer cell; some seem to be attached to the Kupffer cells, but the majority are free in the capillaries. Cells exactly like these may be found singly, or in nests lying in hollows of the liver cells below the endothelium. Individual cells of such a nest may be in mitosis. The Kupffer cell overlying the nest may also be in mitosis. The similarity between the cells of the nests and the large unclassified cells in the liver capillaries on the one hand, and the similarity and anatomical relation of both types to the dividing young Kupffer cells on the other hand are striking.

The nodules are of two types; those occupying the larger portal venules, and those of the intra-acinar capillaries. The first, even at the end of seven days, contain polynuclear leucocytes, though they are much less numerous than at the end of forty-eight hours. Besides these, large giant cells, stellate cells resembling Kupffer cells, and large mononuclear cells, compose the clumps. The vessel wall may or may not be intact in any one nodule. Outside the vessel similar cells may sometimes be found. Fibrin is not demonstrable.

The smaller intra-acinar nodules afford the more satisfactory study, consisting as they do of giant cells, which may or may not preserve their normal relation to the lining of the capillaries. Often the giant cell is pushed out and surrounded on all sides by cells similar to Kupffer cells, and by large mononuclear cells which may show mitosis, and occasionally by lymphocytes. These nodules completely fill the containing vessel, and in the later stages the centers may be caseous.

EXPERIMENTAL TUBERCULOSIS IN VITALLY STAINED RABBITS.

The difficulty of determining the origin of the cells of the young tubercle from ordinary stains is evident from the above description. It is impossible to say what the origin may be, beyond the fact that mononuclear cells are involved. Whether the endothelial or the mononuclear blood elements are concerned cannot be decided. An intermingling of the two cell types has taken place, and every transition from either type to the giant cell can be imagined. The vital stain, free from any possibility of inclusion of the blood cells, should furnish discriminating light here.

Rabbit 98.[3]—Killed twenty-four hours after intravenous injection of 20 c.c. of 1 per cent. solution of trypan blue, and one half hour after inoculation with tubercle bacilli.

Liver.—Aside from slight pigmentation, the cells show nothing abnormal.

The Kupffer cells are enlarged and relatively deeply stained. They correspond in size and intensity of vital staining to those of rabbit 97 A. Mitotic figures are found in much greater frequency than in the animals that received vital stain alone, and Kupffer cells containing two to three or four nuclei, though rare, may be found. The Kupffer cells show phagocytosis.

Blood.—Except for an apparent increase of polynuclear leucocytes there is no change in the blood picture.

Plugs.—Both in the larger interlobular veins and in the intra-acinar capillaries cell accumulations occur. The walls of the veins may be broken and their fibers stained vitally. Within the vein a bluish fibrillar substance occurs, in which many polynuclear leucocytes, a few red blood cells, and an occasional lymphocyte are enmeshed. In the center of these areas clumps of bacteria are frequently found. The surrounding polynuclear cells seldom contain bacteria, but the Kupffer cells frequently contain organisms even in distant areas. These cells may show mitosis, and a small group of cells may accumulate around them. Fibrin is not demonstrable.

Rabbit 100.—Killed thirty hours after intravenous injection of 20 c.c. of 1 per cent. solution of trypan blue, and six hours after inoculation with tubercle bacilli. Clumps of blue staining material scattered throughout the liver are readily seen with the naked eye.

Liver.—The cells contain granules of trypan blue. Many of the Kupffer cells are enlarged. Granules of vital stain are found in them as well as in the normal endothelial cells, but a number of cells are still unstained. Kupffer cells with several distinct nuclei occur occasionally. These may be so large that they almost occlude the lumen of the capillary. Mitoses occur in the Kupffer cells, but are not demonstrable in those with more than one nucleus. Phagocytosis is marked.

Blood.—The capillaries contain little blood. Here and there a few red blood cells occur. Polynuclears may also be seen, but they are not abundant. Some of the mononuclear cells are definite lymphocytes, but others resemble polyblasts. None of the blood cells are pigmented with the vital stain.

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[3] This animal was not injected with formalin after death on account of the risk of washing out the fresh capillary plugs in the liver.

Plugs.—Most of the plugs are small and lie in the intra-acinar capillaries which they may entirely occlude. They consist of large, swollen, pigmented Kupffer cells with varying numbers of large vesicular nuclei, a few polymorphonuclears, and an occasional mononuclear cell. The Kupffer cells are easily identified by their shape and pigment content. Some of them may be still attached to the vessel wall. They may contain bacteria, but this is difficult to demonstrate, since the bacteria are obscured by the vital stain. Here and there a mass of blue staining rods which resemble the bacteria occur. The larger plugs resemble those in the half hour stage.

Rabbit 97 B.—Mar. 10. 20 c.c. of trypan blue injected into ear vein.

Mar. 11. 20 c.c. of trypan blue injected in ear vein and inoculated with tubercle bacilli.

Mar. 12. Killed twenty-four hours after inoculation.

Scattered through the liver tissue are plugs of material conspicuous on account of their blue stain.

Liver.—The cells are normal in appearance and contain granules of trypan blue. The Kupffer cells are increased in size and number and contain many blue granules. The number of granules varies; some have only a few small ones, while others are so densely packed with them that the nucleus is obscured. Mitotic figures occur (figures 1, 2, and 3), and multinucleated cells are much more abundant than in the six hour stage. Phagocytosis is marked.

Blood.—The capillaries are dilated, but contain little blood. Polynuclears and red blood cells occur as usual. Mononuclears are more abundant. Most of them resemble polyblasts, but some are evidently Kupffer cells cut tangentially, and still have a process extending to the wall of the capillary. These have blue granules. Still others are found similar to them in every respect, but not attached to the vessel wall and without pigment granules. These cells are occasionally found lying in indentations of the liver cells below the Kupffer cells. With the exception of one polymorphonuclear leucocyte containing a few pigment granules, the cells of the circulating blood are normal.

Plugs.—These stand out sharply on account of the concentration of vital stain in them. Some of them consist of a single giant cell which is sometimes still attached to the vessel wall, and surrounded by only a few stellate Kupffer cells well laden with pigment granules. The larger plugs show a center of bluish material, in which polynuclear leucocytes and mononuclear cells are found. Surrounding these one or more pigmented Kupffer cells, or mononuclear, polyblast-like cells occur and form the remaining constituents of the plugs.

Bacteria.—A few bacteria are found in the Kupffer cells and in the giant cells. They are, however, made out with difficulty, as they are either stained or overshadowed by the trypan blue.

Rabbit 92.—Mar. 1. Intravenous injection of 21 c.c. of trypan blue.

Mar. 2. Intravenous injection of 21 c.c. of trypan blue. Inoculated with tubercle bacilli two hours later.

Mar. 3. Intravenous injection of 21 c.c. of trypan blue. Animal killed thirty-six hours after inoculation.

Liver.—The section is studded with many masses of vitally stained cells, usually near the periportal spaces. The liver cells are coarsely granular and vacuolated. They contain a few small granules of trypan blue. The Kupffer cells are almost all enlarged and appear more numerous. The greater number of them are stained as usual.

A considerable number of cells with two, three, or more nuclei are found. These may still occupy their positions on the capillary lining. Giant cell formation is particularly marked (figures 5, 6, and 7). Mitoses are found in unusual numbers in Kupffer cells with one nucleus, but not in multinucleated cells.

Blood.—The capillaries contain red blood cells, and polynuclear leucocytes, but the mononuclear cells predominate. Some of them are lymphocytes, but the majority are similar to polyblasts. The latter are not infrequently seen in mitosis.

Plugs.—More numerous than in rabbit 97 B. In the larger ones, the polynuclear leucocytes have to a great extent disappeared. Deeply pigmented giant cells still partially attached to the vessel wall, stellate pigmented and non-pigmented Kupffer cells, and polyblast-like cells occur as usual. The polyblast-like cells in some places seem to push the giant cell away from its attachment to the vessel wall and infiltrate the surrounding tissue to a slight extent. Mitoses occur in these polyblast-like cells and are often demonstrable in the Kupffer cells in the vicinity of the larger plugs. The smaller intra-acinar plugs present the usual picture. They invariably contain a large pigmented multinucleated giant Kupffer cell (figure 8), and in some instances are surrounded only by irregularly pigmented Kupffer cells. The Kupffer cells vary in the amount of their pigment. In other instances polyblast-like cells are associated with the Kupffer cells at the periphery of the giant cells. These cells are not pigmented but sometimes cells almost identical in type contain a few granules. The smaller plugs may entirely occlude the capillary, and in some instances, where the giant cell is still attached in its original position, the lumen of the vessel may be entirely obstructed by it alone.

Bacilli.—Acid-fast involution forms occur rarely in the giant and Kupffer cells, but they are demonstrated with difficulty.

Rabbits 95 and 96.—Mar. 1. Intravenous injection of 21 c.c. of trypan blue.

Mar. 2. Intravenous injection of 21 c.c. of trypan blue, and 10 mg. of tubercle bacilli.

Mar. 3. Intravenous injection of 20 c.c. of trypan blue.

Mar. 4. Killed fifty-four hours after inoculation.

A detailed description of the cases will be omitted. Only the points will be emphasized which have not been noted in the previous experiments.

Liver.—The Kupffer cells show the usual pigmentation, mitosis, phagocytosis, and giant cell formation. One multinucleated Kupffer cell was found in which one of nuclei was in mitosis.

Blood.—The usual cellular elements are present. Polyblastic cells are the most conspicuous. They occasionally show mitosis, and some of them contain a few minute pigment granules. Cells similar to these, but more pigmented, occur singly or in nests in small pockets formed between the Kupffer cells and the liver cells. Individual cells in the nests may be undergoing mitosis. Their direct origin from Kupffer cells was not demonstrable, although large Kupffer cells in their vicinity, in some cases lying over the nest, were seen to be undergoing mitosis.

Plugs.—The larger plugs contain fewer polynuclear leucocytes; the smaller ones are more cellular, otherwise they resemble those described in the previous experiment. In rare instances pigmented Kupffer cells may be so closely packed against a giant cell that the possibility is suggested of a giant cell increasing in size by fusion with Kupffer cells. In one tubercle occurring in a large periportal vein a tubular structure was found lined by swollen, pigmented Kupffer cells; at one end the cells dilated and filled the lumen, giving the appearance of a giant cell.

Rabbit 74.—Jan. 16. Intravenous injection of 14 c.c. of trypan blue.

Jan. 17. Intravenous injection of 20.5 c.c. of trypan blue.

Jan. 18. Intravenous injection of 20.5 c.c. of trypan blue and 10 mg. of tubercle bacilli.

Jan. 19. Intravenous injection of 20.5 c.c. of trypan blue.

Jan. 20. Intravenous injection of 20.5 c.c. of trypan blue.

Jan. 21. Killed seventy-two hours after inoculation with tubercle bacilli. The giant cells are particularly numerous and stained brilliantly with the vital stain. They are so often attached to the vessel wall that where this is not found it may be attributed to the plane of the section. One giant cell had three nuclei, one of which was undergoing mitosis. The polynuclear leucocytes in the circulating blood rarely contain a few blue granules. Kupffer cells are found free in the capillaries; they may retain their usual shape, or may be rounded with only a single process connecting them with the capillary lining. Such cells may be pigmented, and are hence distinguishable from the polyblastic cells.

Plugs.—In one of the plugs a non-pigmented giant cell was found. Pigmented and non-pigmented Kupffer cells, indistinguishable from polyblasts, may be found free in the capillaries. They may have several nuclei and the folding of the nucleus suggests an increase by budding. It is possible that these Kupffer cells are young, and the transition forms suggest that they may be the source of non-pigmented giant cells.

Experiments were carried on similar to those described in which the animals were killed at varying intervals up to eleven days. No new facts were brought out, however, and consequently a detailed description will not be given.

Rabbit 44.—A chronically stained animal had received the usual dose of trypan blue at regular intervals from Nov. 21 to Feb. 9, 160 c.c. of dye being injected during this period. On Feb. 15 the animal was inoculated with tubercle bacilli and on Feb. 19 a lobe of the liver was excised for study.

Liver.—The cells show the usual picture found in chronically stained animals. Large numbers of deeply stained macrophages occur throughout the liver capillaries. Definite tubercles were found in considerable numbers, usually located near the periportal areas. These are composed of central giant cells, and epithelioid and mononuclear cells. Occasional polynuclear leucocytes are found in the larger clumps. The point of chief interest, however, is that the tubercles contain practically no vital stain. The small amount of stain that is found in the tubercle appears as fine granules in the giant and epithelioid cells.

This case (rabbit 44) seems to us of interest from two points of view. First, the contention that preëxisting wandering cells form the tubercle is decisively disproven. There existed in this animal a great number of large free wandering cells electively stained so that their participation in the tubercle would have given us stained cells there. Secondly, the experiment demonstrates that the vital dye must be present in quantity in the body fluids so as to be accessible to the new growth at the time of its formation. All our studies have shown the electivity of the tubercle for the vital stain, yet in rabbit 44, where no free stain was available, the tubercles were almost colorless. That failure to stain them vitally was not due to their leucocytic nature or to any refractility to the vital dye was shown by the experiments begun as was rabbit 44, but terminated by a vital dye of another color.[4] In these experiments red tubercles could be grown in blue livers.

Summary.—Within half an hour after the inoculation of tubercle bacilli in vitally stained animals cellular accumulations are found, particularly in the region of the periportal vessels and in the intralobular capillaries. The tubercle bacilli are found in clumps free in the centers of the larger cellular masses, but even in the early stages a large number of organisms are found in the bodies of the cells. The polynuclear leucocytes contain relatively few bacteria, but many of the Kupffer cells, even in areas remote from the cellular accumulation, contain organisms. In the later stages the clumps of free bacilli become less conspicuous, and organisms are demonstrable only in the bodies of Kupffer cells. The blue vital stain interferes somewhat with the demonstration of the organisms for it is very intense in the case of cells containing the bacilli. At the end of thirty minutes the plugs in the large vessels are partly composed of a homogeneous blue staining material in which are large numbers of polynuclear leucocytes. Occasional red blood cells and lymphocytes may be found here, but fibrin is not demonstrable. The vessel wall stains vitally, and this is probably the result of an injury brought about by the presence of the organism. The polynuclear leucocytes in the clumps gradually disappear and are replaced by cells which will be described below. At the end of half an hour mitotic figures are relatively frequent in the Kupffer cells, and this process continues through all the stages studied. As a result, Kupffer cells are found at the end of six hours with two or more nuclei, and at the end of twenty-four hours and later stages large brilliantly pigmented multinucleated giant cells lying in the position of the Kupffer cells form one of the most conspicuous elements in the section. The large Kupffer cells and giant cells[5] usually contain bacteria, and it is especially around these cells that the formation of the miliary tubercle may best be studied. The giant cell may lie partially or entirely free in the capillary connected only by a streamer to the lining cells. It is stained intensely vitally and, as a rule, it is surrounded by a varying number of cells which may all be stellate or wing-shaped Kupffer cells, containing more or less blue pigment. In this way a miliary tubercle may be found composed entirely of pigmented cells. Some of the Kupffer cells are not pigmented, in which case they are recognizable only by their peculiar form. This involves the separation of the non-pigmented cells from a third type of cell which closely resembles the polyblast. This cell is frequently found in the capillaries, and seems to occur in greater numbers after the six hour stage. Mitotic figures are found in them. They also occur singly, or in nests lying between the Kupffer cells and the liver columns. As a rule, they are not pigmented, but occasionally a few fine granules of blue may be seen in the protoplasm. The cells occur abundantly in the larger tubercles.

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[4] We employed dyes whose usefulness in this respect was discovered by Evans and Crowe (*Bull. Johns Hopkins Hosp.*, 1914 (in press)). Diamine fast scarlet 10 BF is a brilliant red which can be fixed in the tissues in formalin.

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[5] The tubercular giant cell noted by Rokitansky and Virchow, and emphasized by Langhans, Wagner, and Schueppel, has caused much discussion, particularly as to whether it is unicellular or multicellular in origin. Weigert, Straus, and Oppenheimer advocate the first theory; Kostenitsch and Wolkow, Kockel, Miller, and Watanabe consider that it results from a confluence of proliferated fixed tissue cells, and Metchnikoff, Borrel, and Wallgren think that it results from the fusion of white blood cells. Metchnikoff, however, has noted the formation of giant cells in the liver of *Spermophilus citillus* ("Ziesel") by a budding of the nucleus. Besides the true giant cells, Weigert, Arnold, Kochel, and others describe pseudogiant cells brought about by plugging of small vessels or bile ducts with exudate.

The nuclei of the large Kupffer cells are polymorphous and frequently several almost independent lobes may be found connected by slender strands of nuclear material. It seems probable, therefore, that the nuclei of the giant cells increase by direct division. Rarely pictures occur which suggest that the giant cells may increase in size by fusion with neighboring Kupffer cells. In the center of the larger tubercles deeply pigmented Kupffer cells closely approximated to the giant cell also suggest the possibility of this fusion. Consequently from the evidence that we have we should not deny the ability of more or less separate Kupffer cells to coalesce more perfectly to form the giant cells. But the endothelium of the hepatic capillaries is normally a common syncytial mass, and giant cell formation seems to us to be probably an intense unicellular response rather than an agmination phenomenon. A series of stages based on the unicellular hypothesis can be found in the growth of the young giant cell, although as a whole this question is of subsidiary interest compared with the manner of origin of the tissue.

Tuberculosis produced in chronically stained animals, in which no trypan blue was present in the circulation at the time of inoculation, resulted in the production of typical non-pigmented miliary tubercles. This is of interest because in chronically stained animals large numbers of deeply stained macrophages are always found in the circulation. These old macrophages are, however, seldom if ever concerned in the young tubercles that arise.

SUMMARY AND CONCLUSION.

In our study of the histogenesis of the miliary tubercle developing inside the liver lobule in animals that have been stained vitally while inoculated with bovine tuberculosis, the controls enable us to recognize the manner in which the vital stain affects the liver. There is therefore no possibility of confusing the effects due to the organism with the effects due to the dye. It is, however, of interest to note that the effects are closely related. The vital stain alone is able to produce gradually some of the same changes that occur with far greater rapidity in experimental tuberculosis. Although in a few hours the Kupffer cells of tuberculous animals begin to react to the disease, in the case of normal animals stained vitally they do not do this until after the third or fourth dose of successive daily injections. After many days, nevertheless, the vital stain alone produces enlargement, proliferation, and separation of Kupffer cells so that these are converted into large free phagocytes which may possess one or several nuclei. These are the gigantic macrophages of chronically stained animals. In all our experiments we have used only acutely stained animals, so that the effects of the dye itself are never sufficient to produce the changes. In fact there is no evidence that the dye accentuates the changes appreciably during the time involved in the experiment. The dye, however, shows us the type of the cells entering into the tuberculous granuloma, for when fed to the body fluids in abundance trypan blue finds its way into all cells capable of receiving it. The vital stain is, as it were, a physiological test for the cells. Whatever the fundamental nature of the vital stain produced by trypan blue and the benzidine dyes may be, it is important that this reaction does not occur to any appreciable extent with mononuclear blood cells, and that it does occur emphatically in the case of the hepatic endothelium. By means of this vital test, then, the following phenomena occur when suspensions of tubercle bacilli are let into the portal blood stream. The organisms, swept on by the blood stream, finally lodge in the terminal branches of the portal vein, where they plug the vessels and continue to multiply. They injure the vessel wall and cause around them an exudative inflammatory process, and finally lead to the formation of tubercles situated not only in these areas but also within the liver lobule. The injury to the vessel wall is manifested in the early stages by the presence of vitally stained areas in its structure. The bacteria at the end of half an hour are found to be extracellular in clumps in the larger vessels, but already to some extent in the bodies of vitally stained Kupffer cells throughout the liver. Exudative inflammation manifests itself by the presence of a transitory accumulation of polynuclear leucocytes about the bacterial clumps, which may be seen as early as half an hour after the inoculation. They continue to be present in the larger cell clumps of the periportal areas for many days, but they are rapidly replaced by other cells, mononuclear in type, so that within a day the histological appearance of the portal plug has changed radically. The mononuclear cell thus entering most actively into the reaction is endothelial and not hematogenous in origin, the vital stain enabling us to make a clear distinction. This fact, evident in the portal plugs, is decisively shown in the case of tubercles developing within the liver lobule. Such tubercles probably result from the localization of individual organisms within the Kupffer cells, for the initial stages of such a probable cycle have been found by us. They consist of the occurrence of mitoses in certain Kupffer cells where the Ziehl-Nielson method shows a bacillus or several bacilli to have been phagocytized (figure 4). Rapid growth of the infected cell now takes place, and at thirty-six hours the multinucleated giant cell produced is largely separated from the other endothelium of the vessel wall. Many bacilli exist within the protoplasm of these cells (figure 5), which are especially distinguishable by their intense reaction to the vital stain. They have received trypan blue to such an excess that low power views of liver sections at the thirty-six hour stage show these cells as deep blue spots (figure 8). The origin of the giant cell from the Kupffer cell is evident not only from the above sequence and from the elective stain, but also from the fact that even when fully formed, protoplasmic strands still join it to its mother tissue,—the normal endothelium of the vessel. The strands entangle other cells in their meshes, especially mononuclear blood cells, one of which, of the polyblastic type, has homogeneous protoplasm and is not infrequently encountered in mitosis. These cells are unquestionably of importance in the lesion of tuberculosis. We have seen them abundantly in the capillaries soon after the inoculation and they also occur singly or in nests between the Kupffer cells and liver columns. They are, as a rule, free from the vital dye. They continue to be concerned in the further growth of the tubercle and with the connective tissue cells make the structure of older tubercles relatively complex. On the other hand, little complexity occurs in the structure of the young intralobular masses. The miliary tubercle formed at the end of thirty-six hours is composed of a giant cell, surrounded by epithelioid cells and by blood cells of the above polyblastic type. The giant cell and its so called epithelioid cells are electively stained and are exclusively derived from the hepatic endothelium.

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EXPLANATION OF PLATES.

PLATE 36.

FIGS. 1, 2, and 3. Three stages in the mitosis of Kupffer cells from the liver of a rabbit twenty-four hours after inoculation with bovine tubercle bacilli. The large stellate endothelial cells are densely laden with the vital blue granules of trypan blue, 40 c.c. having been given intravenously in two doses. The section is counterstained with aqueous cochineal.

PLATE 37.

FIG. 4. Kupffer cell from the same rabbit as figures 1, 2, and 3. The section is stained by the Ziehl-Nielson method and shows the body of a Kupffer cell in mitosis containing three bacilli. The presence of these might be considered the immediate stimulus for growth of the cell.

FIG. 5. Young endothelial giant cell from the liver of a rabbit thirty-six hours after inoculation with bovine tubercle bacilli, stained as in figure 4 by the Ziehl-Nielson method. The cell is still connected to the vessel wall by protoplasmic processes; within it many bacilli and vital blue granules are found; four nuclei are included in the section; mitosis of any of the nuclei in the giant cell is rarely seen, so that direct division probably occurs.

PLATE 38.

FIGS. 6 and 7. Young endothelial giant cells from the liver of the same case as figure 5. The counterstain is aqueous cochineal. The enormous engorgement of these cells with the vital blue is readily seen. As yet no epithelioid cells surround the giant cells. The giant cell of figure 7 is already almost free, but that shown in figure 6 is still part of the endothelial wall, the nuclei of which had increased rapidly, so that a syncytial mass was formed.

PLATE 39.

FIG. 8. Low power view of a thirty-six hour old tubercle, showing the remarkable increase of the vital stain in the tubercle cells as compared with the remaining tissue. The tubercle is still almost purely the central giant cell, though several future epithelioid cells are noticeable.

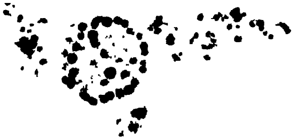

FIG. 1.

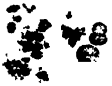

FIG. 2.

FIG. 3.

(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)

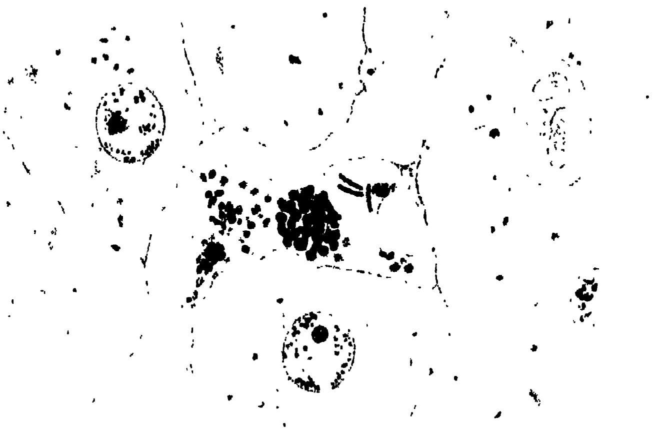

FIG. 4.

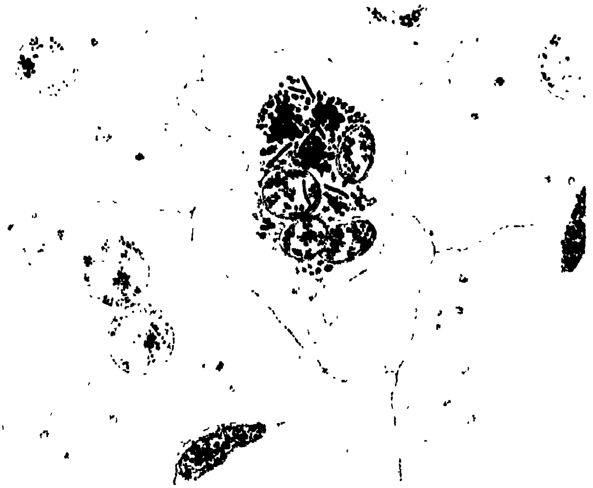

FIG. 5.

(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)

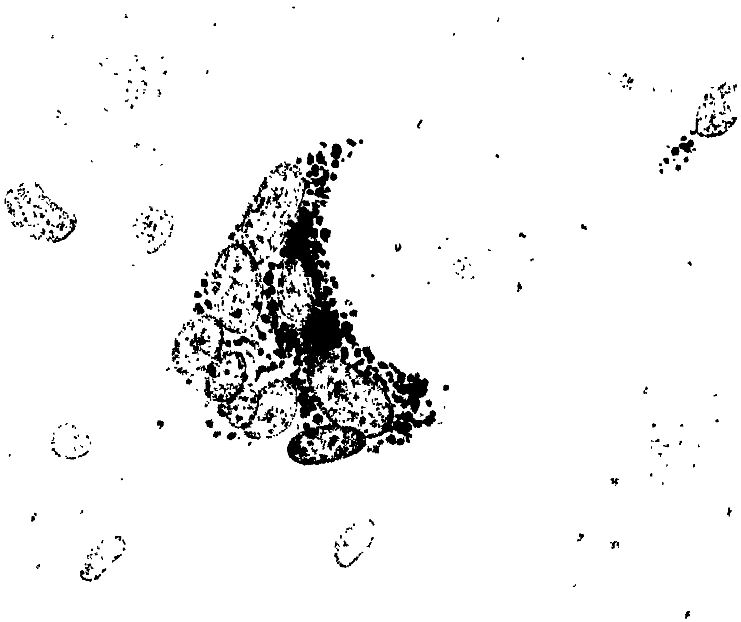

FIG. 6.

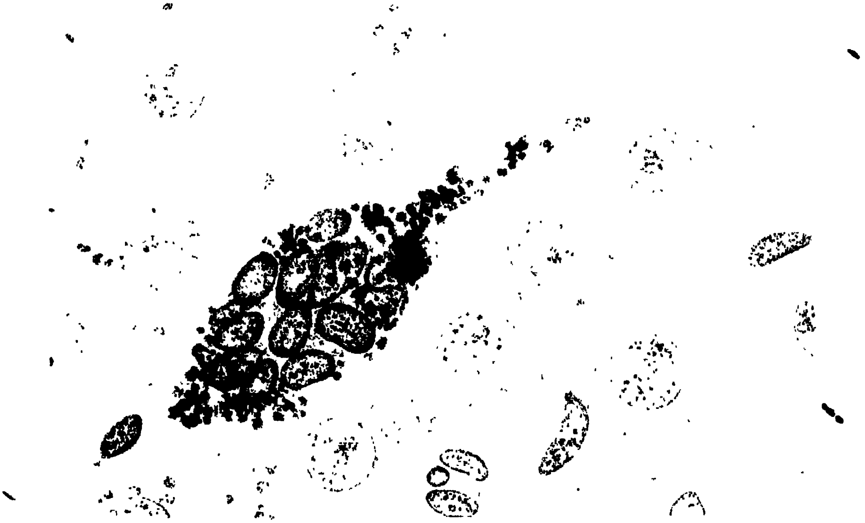

FIG. 7.

(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)

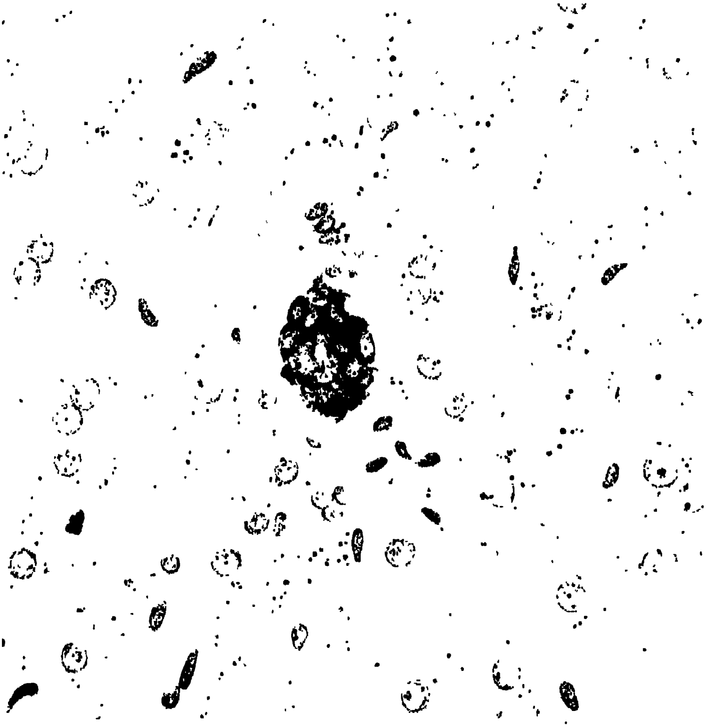

FIG. 8.

(Evans, Bowman, and Winternitz: Miliary Tubercle in Rabbits.)

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ON THE APPLICATION OF CERTAIN CULTIVATION METHODS TO THE STUDY OF INFECTIOUS DISEASES.

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The chief recent advances in our knowledge of pallida have come from the recognition of two new classes of pathogenic microörganisms, namely, the Spirochaetes and the filterable viruses. In both instances knowledge of the relation of the microörganisms to their special diseases has been acquired, not according to the classic methods of Koch, whose postulates could not be fulfilled, but by means of other criteria, which while perhaps less stringent, have none the less led to valuable results.

One of the difficulties in the way of the fulfillment of Koch's postulates arose from the impossibility of cultivating artificially these classes of parasites by the means hitherto employed for artificial cultivation of microörganisms. Hence it became necessary to search for new methods that would set aside this initial obstacle. At present it may be said that we possess methods by which artificial cultivation of the Spirochaetae and of certain minute and filterable organisms may be secured. With this achievement a second difficulty became apparent, namely, that when the Spirochaetae are thus cultivated the strictly parasitic species, e. g., Treponema pallidum, Treponema pertenue, Treponema gallinarum, tend as a rule to lose virulence, so that it is impossible in many instances to produce infection of animals with them. Luckily in the Spirochaetae the morphology is a valuable guide in the identification of species and this class is subject also to determination by means of immunity reactions. With our slighter knowledge of the cultivated filterable viruses we are not permitted to generalize, but it appears also that the minute organism cultivated from poliomyelitic tissues, as well as the organism cultivated from rabic materials, gives less constant pathogenicity than the viruses derived directly from infected animals.

It is my intention in this paper to restate the methods of cultivation employed for the Spirochaetae and for poliomyelitic and rabic tissues. It seems appropriate in view of the fundamental contributions to the subject of the Spirochaetae and their therapeutic control made by Professor Ehrlich that in a publication in his honor this subject should be treated. Besides this, the cultivation of these classes of microörganisms possesses at present a large inherent interest and, moreover, the methods themselves as at present perfected require such painstaking attention to detail in order to insure fairly uniform results that it seems proper to repeat and emphasize their nature. In the hands of the experienced the methods can be applied with more certainty of success; but the beginner in their use, even if a highly skilled bacteriologist, will have to approach the cultivation of highly parasitized species such as Treponema pallidum and the globoid bodies of poliomyelitis with the understanding that many failures may precede successful results.

To begin with the Spirochaetae, the most important of which is Treponema pallidum, the method employed is based upon the anaerobic principle of Theobald Smith and consists in the employment of suitable animal tissues with suitable nutrient media, etc. My first experiments were made with the syphiloma of the testicle of the rabbit. After many failures and the substitution of the medium just mentioned, one positive tube out of twelve, and that one the last to be examined, was eventually obtained. It may be of interest to state that at the same time that the particular strain mentioned was submitted to cultivation four other strains distributed in forty-eight tubes forming a part of the same series were subjected to cultivation, none of which gave any growth. This detail is emphasized merely to indicate how delicate the adjustment must be to secure initial success. After this slight forward step ten different strains of the pallidum were tested for six months, of which six were finally secured in pure culture while the remaining four never yielded to cultivation. After the initial cultivation of the six each strain had to be tested repeatedly before permanent cultures were obtained. In endeavoring to apply these results to the cultivation of the pallidum directly from syphilitic lesions it was found that the method was not applicable, for the reason that when they occur in testicular lesions the strains are nearly pure, but when they are obtained from pure lesions they are for the most part grossly contaminated. This difficulty was finally overcome by employing a solid medium based on the same principle as the fluid medium and also containing fresh animal tissues. In the course of another year six strains of the pallidum were cultivated directly from human sources. In spite of certain modifications in technique introduced into the method it is still as difficult to obtain fresh cultures of the pallidum as it was at the beginning of the study three years ago.

The same facts apply to the cultivation of Spirochæta pertenuis, which in spite of many attempts has been successfully cultivated once only. The experience in the cultivation of the pallida and pertenuis facilitated the cultivation of the mouth Spirochaetae. The latter being saprophytic could be secured readily by means of the solid medium and in that way three different species were obtained (macrodentium, microdentium, mucosum). A comparison of the various species of Spirochaetae now cultivated established the fact that the pallidum never produces a disagreeable odor such as is produced by some of the mouth Spirochaetae. Similarly, through the use of the solid medium several saprophytic Spirochætae (refringens, calligyrum, phagedenis) were obtained in pure culture from the region of the human genitalia.

Up to the present the different Spirochaetae mentioned require strict anaerobic conditions for growth in artificial media and the pathogenic species especially demand also the presence of fresh animal tissues. These fresh tissues serve as a source of nutrition as well as consumer of oxygen. However, the presence of the fresh tissues in a fluid medium does not enable the pallidum or pertenuis to grow except when the cultures are enclosed in an anaerobic apparatus.

In endeavoring to cultivate the Spirochaetae of relapsing fever and fowl spirillosis it was ascertained that they also require the presence of fresh tissue, but in addition demand a trace of oxygen. While in a sense these Spirochaetae are anaerobic they yet require a minimal oxygen tension in the medium in order that growth may occur. Moreover, with these Spirochaetae the origin of the ascitic fluid makes a great difference, since a sample suitable for one variety appears unsuitable for the other. Hence the selection of the samples of ascitic fluid plays an important part in the cultivation of these parasites. I found that the European variety of recurrens was the most fastidious in this respect; and Hata[1]) has recently shown that this difficulty may be eliminated by a method which he has perfected.

With the experience gained in the cultivation of the Spirochaetae attention was directed to the class of parasites grouped under the name of filterable or ultra-microscopic organisms, which were subjected to cultivation experiments in accordance with the principles which had now been learned.

A general plan for carrying out cultivation experiments follows.

The material for the purpose of cultivation is first introduced into the tubes which already contain a fragment of fresh tissue from a suitable animal. After the inoculation of the tubes the following nutritive substances are added in sufficient quantities (10–12 cm. deep):

Four tubes for each, two of which are covered with paraffin oil.	a) Selected ascitic fluids (several specimens to be tried).
	b) Pure plasma diluted with Ringer solution (the plasma from suitable animals).
	c) Ascitic agar (1 : 2).
	d) Serum water.
	e) Ringer plasma agar (1 : 2).
	f) Serum with pepton, albumoses, amino acids, etc. (to be tried in turn).
	g) Serum with carbohydrates (different sugars to be tried in turn).

Two tubes, each one of which is covered with paraffin oil.	h) Bouillon (control).
	i) NaCl solution (control).

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1) Hata, Zbl. f. Bakt., Abt. I, Orig., 1913, Bd. 72, H. 1 u. 2.

The above is made in duplicate and one series is put in an anaerobic apparatus, while the second is placed in a thermostat without the anaerobic apparatus.

According to the conditions which are found necessary for artificial cultivation we may introduce the following classifications:

1. Strictly anaerobic; necessity of the presence of suitable animal tissue, and suitable nutrient media; shows great preference for a fluid medium. Initial growth is possible in a solid medium when contaminating bacteria are present, otherwise not. To this group belong pathogenic tissue Spirochaetae: Treponema pallidum and Treponema pertenue.
2. Same as the above, except that a culture may often be obtained without an anaerobic apparatus. No initial growth in a solid medium. To this group belong the viruses of poliomyelitis and rabies.
3. Same as the above, except that no culture can be obtained unless a certain amount of oxygen is allowed to be present. No growth in a solid medium. To this group belong the blood spirochaetae of relapsing fevers, tick fever and fowl spirillosis.
4. Strictly anaerobic; necessity of suitable nutrient media. Initial growth is possible in a solid medium when impure; the presence of fresh animal tissue favors the growth, but is not essential. To this group belong the saprophytic spirochaetae: Treponema macrodentium, Treponema microdentium, Treponema mucosum, Treponema calligyrum, Treponema refringens, etc.

It has also been found that the modification of certain nutrient fluids (ascitic fluids) by means of heating, filtration or addition of some extragenous substances (pepton, sugars, etc.) renders impossible the initial growth of pathogenic spirochaetae, as well as the viruses of poliomyelitis and rabies, although the addition of pepton or sugars to the media does not interfere with the growth of these organisms (with the exception of the virus of rabies) which had been cultivated on artificial media for many generations.

Another fact of importance is that the pathogenic tissue spirochaetae (pallidum and pertenue) and the virus of poliomyelitis, can not be grown on a solid medium until after they have been adapted to the fluid media for many generations. The virus of rabies still remains refractory to the cultivation on a solid medium. During the first few generations of the poliomyelitic virus in a fluid medium it is difficult to demonstrate the globoid bodies on account of the scarcity of their number and the difficulty in staining.

I desire now to repeat what I mentioned at the outset, namely, that in this article I have endeavored to set down again not only the details of the methods which have been employed in the cultivation of the Spirochaetae, the globoid bodies of poliomyelitis, and certain microörganisms from rabic materials, but also to emphasize that the methods as at present perfected are more difficult of application than the common methods employed in bacteriology; and that painstaking attention to details and many repetitions are likely to be required before the methods are successfully mastered.

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A NOTE ON THE PATHOGENICITY OF TRYPANOSOMA LEWISI.*

BY WADE H. BROWN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In the usual scheme of classification of trypanosomes, *Trypanosoma lewisi* occupies the position of the type species of a number of non-pathogenic trypanosomes.[1] While this usage is justifiable in the present state of our knowledge of these organisms, one must not lose sight of the fact that there is abundant evidence to show that *Trypanosoma lewisi* is not strictly non-pathogenic, but occasionally manifests a decided virulence for rats, especially young ones. Apart from such frequent disturbances as fever, anemia, and loss of weight, a considerable mortality may occur among infected rats. Perhaps the best instance that can be cited is that reported by Jürgens[2] who noted a mortality of 29.3 per cent. (16 out of 47) among young rats. Other authors have noted a slight mortality, or no mortality, resulting from infections of *Trypanosoma lewisi*. These differences in pathogenicity indicate that there are strains that differ fundamentally as regards their virulence. Delanoë[3] has added support to this conception of pathogenic and non-pathogenic strains of *Trypanosoma lewisi* by showing that while certain strains, or organisms from certain sources, are incapable of infecting mice, other strains may infect even a considerable percentage (40 per cent.) of the mice inoculated. Further, Roudsky[4] has shown by his "reinforced virus" that the virulence of a given strain is not absolutely fixed, but that it can be markedly increased for both rats and mice. Finally, Wendelstadt and Fellmer[5] have succeeded in

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* Received for publication, February 15, 1914.

[1] Laveran, C. L. A., and Mesnil, F., Trypanosomes et trypanosomiases, 2d edition, Paris, 1912, 241.

[2] Jürgens, *Arch. f. Hyg.*, 1902, xlii, 265.

[3] Delanoë, P., *Compt. rend. Soc. de biol.*, 1911, lxx, 649.

[4] Roudsky, D., *Compt. rend. Soc. de biol.*, 1910, lxviii, 421, 458; 1911, lxx, 741.

[5] Wendelstadt, H., and Fellmer, T., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 422; 1910, v, 337.

raising the virulence of *Trypanosoma lewisi* by passage through cold-blooded animals.

These facts suffice to show the existence of pathogenic strains of *Trypanosoma lewisi* and indicate that possibly all strains possess potential pathogenic properties. Unfortunately, the known facts regarding its pathogenicity are too meagre to warrant any generalizations. Since the natural host of this organism makes it peculiarly suited to laboratory study, further work upon this subject seems highly desirable, as a clearer comprehension of the conditions governing its pathogenicity would probably aid materially in advancing our knowledge of the more important group of organisms,—the pathogenic trypanosomes.

Recently, we have had under observation a strain of *Trypanosoma lewisi* that, for a short time, showed an unusual increase in its virulence, and an account of the action of this organism is offered as a contribution to the study of its pathogenicity.

A complete genealogy of this strain of *Trypanosoma lewisi* is not available. The organism was isolated from a natural infection in a wild rat and has been carried for several years in white rats with no unusual manifestations of virulence. In October, 1913, for three successive generations, fatal infections resulted from the intraperitoneal injection of one to two drops of tail blood, diluted with one cubic centimeter of a 1 per cent. sodium citrate solution, death taking place eight to eleven days after inoculation. Five rats out of the six inoculated succumbed to this infection. At first it appeared improbable that *Trypanosoma lewisi* was entirely responsible for such an unusually high mortality, but as careful investigation, including cultures from the peritoneal cavity and heart's blood, failed to reveal any other cause, the following test of virulence was applied.

Experiment 1.—Nov. 28, 1913. A large rat infected with strain I of *Trypanosoma lewisi* was bled to death on the twelfth day of infection and the blood defibrinated. At this time there was a heavy blood infection with a few multiplication forms still present. The rat was weak and showed a marked anemia and loss of weight. With aseptic precautions, ten normal rats were injected intraperitoneally with 0.2 c.c. of blood diluted with 0.8 c.c. of an 0.85 per cent. salt solution. Five of the rats weighed between 100 and 170 gm., and the other five between 40 and 70 gm. As a control, four normal rats (two large and two small) of the same lot were kept under observation and two large immune rats were injected intraperitoneally with 0.5 c.c. of blood in 1 c.c. of salt solution.

Nine of the normal rats inoculated showed trypanosomes in their blood within twenty-four hours. The tenth rat, a large one, showed a very few organisms at forty-eight hours, but the infection was transient, disappearing completely within five days without any demonstrable multiplication having taken place; this rat was probably an immune. Another large rat died in forty-eight hours and the autopsy showed a marked bronchiectasis. If we exclude these two rats from the list of normal ones, the eight remaining rats showed a rapid increase in trypanosomes in the blood with definite multiplication on the third day, persisting until the fatal termination of the infection. The rats became torpid and weak, with marked anemia and dyspnea, and slight loss of weight. A few rats showed bloody nasal and lachrymal discharges, and all of them developed a diarrhea with greater or less abdominal distension. Two of the large rats died on the sixth day, and the third died on the seventh day, while all five of the small rats died on the ninth day after inoculation.

Autopsies on these rats showed an acute enteritis, marked splenic enlargement and hyperplasia of the bone marrow, and a few foci of necrosis in the liver. Several rats showed a moderate pulmonary congestion but no bronchiectasis or pneumonia. Cultures made immediately after death from the peritoneal cavity and from the heart's blood of three rats remained sterile. No cultures were taken from the other rats.

The normal and immune controls remained unaffected for one month, when observations were discontinued.

The existence of nasal and lachrymal discharges and diarrhea in these rats would naturally incline one to suspect some condition complicating the trypanosomiasis. In an attempt to clear up the relation of these conditions to the trypanosomal infection, a number of observations have been made on normal and immune rats inoculated with different strains of *Trypanosoma lewisi.* These observations show that the above conditions may recur frequently in a given series of inoculations while they do not appear in control immunes inoculated from the same source, in uninoculated normal rats from the same lot, or in rats inoculated with another strain of *Trypanosoma lewisi.* Likewise, normal and immune rats kept in contact with infected rats showing these conditions, with a single exception, have not developed these symptoms except as the normal rats became infected with trypanosomes by natural means. The fact that the same symptoms occur in the course of other trypanosomal infections in the rat indicates that they may be a part of the morbid manifestations of trypanosomiasis. Whether these conditions are the result of an uncomplicated trypanosomal infection or the result of a latent infection whose development is favored by the trypanosomiasis is not clear. In either case, however, it is evident that it is the trypanosome that is directly or indirectly responsible for the condition.

The series of fatal infections in the stock transfers of this organism was interrupted by inoculating two young rats weighing seventy grams, with one drop of tail blood, taken on the tenth day of infection, from the rat killed for the above experiment. One of the rats was extremely ill for about twenty days but finally recovered. The infection in the other rat was much less severe. On the next transfer from the first of these rats, one large and one small rat were inoculated and the resulting infections were still further decreased in severity.

The small rat of this series was then sacrificed for a second test of the virulence of this strain of *Trypanosoma lewisi,* in this instance contrasted with another strain, recently isolated from a natural infection in a white rat.

Experiment 2.—Dec. 17, 1913. A young rat infected with strain I of *Trypanosoma lewisi* and another young rat infected with strain V were bled from the heart on the eighth day of infection and the blood was collected and defibrinated under aseptic conditions. The blood of the two rats showed about an equal number of trypanosomes with many multiplication forms. From each of these rats, five rats weighing 80 to 90 gm. were inoculated intraperitoneally with 0.2 c.c. of blood diluted with 0.8 c.c. of salt solution.

The five rats infected with strain I all showed an incubation period of less than twenty-four hours. Three of the five rats developed a severe infection with weakness, a high grade anemia, and loss of weight. The other two showed only a moderate infection. None of the rats died and trypanosomes were present in the blood of four on the thirtieth day, when observations were discontinued. Of the five rats infected with strain V, the incubation period of four was less than twenty-four hours, of the fifth between thirty-six and forty-eight hours. Only one of these rats showed any appreciable disturbance from the infection. This rat showed an extreme blood infection, with weakness, torpor, anemia, and loss of weight, persisting for about three weeks. Trypanosomes disappeared from the blood of two of these rats within twenty days and from a third by the twenty-sixth day.

Although none of the ten rats died from the infection, the difference in the severity of the infection produced by the two strains of *Trypanosoma lewisi* was sufficiently well marked to show that strain I was decidedly more virulent than strain V.

This series of observations lends support to the idea that there are strains of *Trypanosoma lewisi* that differ fundamentally as to their pathogenicity, but what is of even greater importance is that any particular strain is subject to marked fluctuations of virulence. From these facts it would seem important to determine the conditions that give rise to such alterations of virulence as have been described, the extent to which these variations of virulence more or less permanently modify the characteristics of the strain, and, finally, whether there are differences in morphology corresponding with variations in virulence or with the differences in pathogenicity exhibited by different strains of *Trypanosoma lewisi.* These subjects are now under investigation.

CONCLUSIONS.

1. Some strains of *Trypanosoma lewisi* may, at times, produce rapidly fatal infections in a large percentage of the rats infected.
2. In such strains of *Trypanosoma lewisi,* a sufficient degree of pathogenicity may persist to warrant the designation of these strains as pathogenic.
3. The pathogenicity of a given strain of *Trypanosoma lewisi* is not constant, but is subject to marked and even sudden variations.

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PENETRATION OF THE VIRUS OF POLIOMYELITIS FROM THE BLOOD INTO THE CEREBROSPINAL FLUID.*

BY SIMON FLEXNER, M.D., AND HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

An active poliomyelitic virus readily causes infection in monkeys when introduced into the brain, subarachnoid spaces, or peripheral nerves. When, however, the virus is injected into the circulation, far greater quantities are required to produce infection and the onset of the usual symptoms and the paralysis is delayed.[1] Were the virus withdrawn from the blood directly by the tissues of the central nervous system neither the greater dose nor the longer incubation should be necessary. It appears that the extranervous organs retain but little of the virus; hence we may suppose that the route by which the virus contained within the blood reaches the central nervous system is an indirect one.

It is now generally conceded that the poliomyelitic virus enters the human body by way of the upper respiratory passages, and in particular through the nasopharyngeal mucous membrane. Once within this membrane the virus may pass through the lymphatic channels surrounding the filaments of the olfactory nerve to the leptomeninges where it reaches the cerebrospinal fluid, or it may first enter the blood and be conducted to the central nervous organs by the general circulation. Flexner and Clark[2] have shown experimentally that when the virus is introduced into the upper nasal mucosa in monkeys its propagation can be followed from the olfactory lobes of the brain to the medulla oblongata and spinal cord. Had the distribution of the virus taken place by way of the general circulation the several parts of the nervous organs should have been

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* Received for publication, February 26, 1914.

[1] Clark, P. F., Fraser, F. R., and Amoss, H. L., *Jour. Exper. Med.*, 1914, xix, 223.

[2] Flexner, S., and Clark, P. F., *Proc. Soc. Exper. Biol. and Med.*, 1912–13, x, 1.

rendered infectious almost simultaneously. The virus of poliomyelitis has hitherto been regarded as strongly neurotropic; but it does not follow from the fact of this neurotropic affinity that the nervous tissues can under all circumstances remove the virus from the blood.

Another possibility exists, namely, that the virus introduced into the blood finds its way not directly to the nervous organs, but indirectly by way of the cerebrospinal fluid. When, therefore, a quantity introduced into the blood is insufficient to cause infection, although a much smaller quantity produces infection when introduced into the brain, the reason may be that the intact choroid plexus prevents the virus from reaching the cerebrospinal liquid. It is well known that this anatomical barrier excludes from the cerebrospinal fluid many substances contained within the blood, that the barrier is not absolute but is capable of being broken down, and that the most frequent source of injury is the pathogenic action of infectious microörganisms.

Hence we may consider that when insufficient quantities of the poliomyelitic virus are introduced into the blood they do not set up poliomyelitis because they fail to injure the choroid plexus; and when poliomyelitis is set up by larger quantities the plexus has been penetrated.

The virus has thus far not been detected by inoculation experiments in the cerebrospinal fluid obtained from human cases of poliomyelitis; and, indeed, when the virus is injected into the subarachnoid spaces in monkeys it remains within the fluid for a limited time and can no longer be detected there at the period of the onset of paralysis.[8] The conditions within the fluid are obviously unfavorable for the propagation of the virus; but the fluid constitutes the most immediate route for the passage of the virus to the interior of the nervous organs in which it multiplies and becomes fixed. The cerebrospinal fluid therefore acts merely as a medium for transporting the virus to the nervous tissues. Hence, should the passage of the virus contained within the blood proceed to the nervous organs by way of the cerebrospinal fluid it should be possible to detect it in transit. From the many failures to discover the virus in the early paralytic stages of poliomyelitis in man and the monkey it might appear that the probability of detecting the virus at all might be small. But it would now appear that when a large dose of the virus has been injected into the blood and a sufficient period allowed to elapse in order that the virus may act upon and injure the choroid plexus, it is possible to detect the presence of the virus in the cerebrospinal fluid by inoculation.

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[8] Clark, Fraser, and Amoss, *loc. cit.*

EXPERIMENTAL.

A passage strain of highly active K virus was employed for the intravenous inoculation. A Berkefeld filtrate of a 5 per cent. suspension caused paralysis and death when inoculated intracerebrally in doses of 0.2 to 0.3 of a cubic centimeter. For the purpose of intravenous inoculation the 5 per cent. suspension was merely centrifugalized and the clear supernatant fluid pipetted off. It was not filtered.

Experiment A. Macacus rhesus 1.—Jan. 30. 250 c.c. of the supernatant fluid were injected into a superficial vein of the leg. No effect was produced by the injection. No symptoms appeared until Feb. 14, when slight excitability was noted. Feb. 16. Weakness of the leg. Feb. 17. Excitability increased and arms paralyzed. Feb. 18. Legs and back paralyzed. Feb. 19. Died.

Lumbar puncture was performed as follows: on Feb. 1 (48 hours after the injection) 0.6 c.c. of cerebrospinal fluid was removed; on Feb. 2 (72 hours after the injection) 0.5 c.c. of fluid removed; on Feb. 3 (96 hours after the injection) 0.9 c.c. of fluid removed; and on Feb. 18 (19 days after the injection) 2.9 c.c. of fluid removed. The several samples of cerebrospinal fluid were free from blood. On Feb. 18 a sample of blood was also taken and defibrinated.

The specimens of cerebrospinal fluid and blood were injected intracerebrally into monkeys as follows:

Macacus rhesus 2.—Feb. 1. 0.6 c.c. of cerebrospinal fluid withdrawn from monkey 1 48 hours after an intravenous injection of K virus was inoculated intracerebrally. No symptoms were produced; the monkey remained well.

Macacus rhesus 3.—Feb. 2. 0.5 c.c. of cerebrospinal fluid withdrawn from monkey 1 72 hours after an intravenous injection of K virus was inoculated intracerebrally. Feb. 14. Monkey somewhat excitable, movements slow. The condition remained stationary until Feb. 25, when the excitability was more marked. No weakness of muscles was detected. Lumbar puncture yielded a clear fluid devoid of globulin and containing forty white corpuscles per c.mm. Feb. 26. Condition not so good; ataxia; slight weakness of both arms. Mar. 10. Condition stationary.

Macacus rhesus 4.—Feb. 3. 0.9 c.c. of cerebrospinal fluid withdrawn from monkey 1 96 hours after an intravenous injection of K virus was inoculated intracerebrally. Feb. 7. Excitability. The condition did not change materially until Feb. 17 when excitability was greater. The condition again became stationary until Feb. 26, when a general paralysis of the muscles was present. Animal etherized. Lesions of poliomyelitis present.

Macacus rhesus 5.—Feb. 18. 2.9 c.c. of cerebrospinal fluid withdrawn from monkey 1 19 days after an intravenous injection of K virus and the 1st day of paralysis were inoculated intracerebrally. Feb. 26, A. M. Excitable; tremor; ataxic. P. M. Left arm flaccid. Feb. 27. Animal prostrate; all members were paralyzed except the tail and a few neck muscles. Etherized. Lesions of poliomyelitis present.

Macacus rhesus 6.—Feb. 18. 5 c.c. of defibrinated blood withdrawn from monkey 1 19 days after an intravenous injection of K virus and the 1st day of paralysis were inoculated intracerebrally. No symptoms developed; the animal remained well.

The experiments bring out several facts. (1) Even a very large dose of an active poliomyelitic virus when injected into the blood produces a much delayed infection. If we consider the certain effective dose of the specimen of K virus employed when injected intracerebrally at 0.2 of a cubic centimeter, then 1,250 doses were introduced into the blood of monkey 1. The average incubation period after an intracerebral inoculation is about 6 days;[4] in monkey 1 the period was 17 days. (2) The cerebrospinal fluid removed 48 and 72 hours, respectively, after the intravenous injection failed, when inoculated intracerebrally, to communicate definite poliomyelitis to rhesus monkeys; while the fluid removed 96 hours (monkey 4) and 19 days (monkey 5) after the intravenous injection caused typical poliomyelitis in monkeys of this species. In monkey 4 the incubation period was indefinite and in monkey 5 it was 8 days. (3) By the 19th day following the intravenous injection of the large dose of the virus and at the onset of the paralysis the virus had disappeared from the blood while it was still detectable in the cerebrospinal fluid by inoculation.

DISCUSSION.

The experiments emphasize in the first place the relatively great difficulty of infecting monkeys with the virus of poliomyelitis by introducing it directly into the blood. At first sight it may appear that this statement is in conflict with the effects of subcutaneous or even of intraperitoneal inoculations. It is, however, not improbable that in all external modes of inoculation practiced, except the intravenous mode, the virus actually penetrates to the central nervous organs by way of the nerves. In any event the difficulties in the way of accomplishing infection through the general blood provide another argument against the notion that the virus of epidemic poliomyelitis is communicated to man by the bite of infected blood-sucking insects.

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[4] Clark, Fraser, and Amoss, *loc. cit.*

On the other hand, the experiments afford valuable support to the hypothesis that infection of the nervous organs in man occurs through the mediation of the cerebrospinal fluid. The virus readily traverses the nasal mucous membrane to reach this fluid, which is capable of carrying the virus to the interstices of the nervous tissues. Apparently the virus enters the intimate structures of the nervous tissues not directly from the blood but indirectly after being passed from the blood to the cerebrospinal fluid. To accomplish this transfer time is required since the barrier of the choroid plexus must first be overcome. At the expiration of 48 hours following the intravenous inoculation the barrier appears still to be intact; at the expiration of 72 hours the passage of the virus seems to have begun, since infection of mild type followed the inoculation of the cerebrospinal fluid removed at this period. At the expiration of 96 hours it appears that the barrier had broken down; and it also appears that under the pathological conditions created the virus, in quantity sufficing to cause infection, still persisted in this fluid as late as the 19th day, although no longer detectable in the blood by the inoculation test. In no other instance has the virus been found in the cerebrospinal fluid at the period of the onset of paralysis.

When we consider the minute size of the microörganism constituting the virus of poliomyelitis we may well wonder at the failure to penetrate the capillaries to gain access to the interstices of the central nervous organs. The case is not wholly unique. von Behring discovered that the hen, which is insusceptible to the action of tetanus toxin injected into the blood, is subject to its effects when introduced into the cerebrospinal fluid. Lesions of the leptomeninges of an interstitial character are implicated in the development of the specific lesions of poliomyelitis. They also suffice to produce at times in man and the monkey a poliomyelitic affection of the meninges in which the central nervous organs proper do not share specifically. Hence the meninges and the cerebrospinal fluid play a highly important and even a determining part in the pathogenesis of epidemic poliomyelitis.

CONCLUSIONS.

The virus of poliomyelitis introduced into the blood may pass indirectly by way of the cerebrospinal fluid to the interstices of the central nervous organs.

To reach the cerebrospinal fluid the virus must first penetrate the barrier of the choroid plexus, which operation requires time. By the inoculation test, no virus was detected in the fluid at the expiration of 48 hours, only small amounts at the expiration of 72 hours, while at the expiration of 96 hours the virus had passed more freely. The virus was still detectable in the fluid at the onset of paralysis 19 days after the intravenous injection.

Pathological conditions of the leptomeninges and the cerebrospinal fluid play an important part in the pathogenesis of epidemic poliomyelitis.

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A STAGE IN THE MIGRATION OF THE ADULT TERTIAN MALARIAL PARASITE. EVIDENCE OF THE EXTRACELLULAR RELATION OF THE PARASITE TO THE RED CORPUSCLE.*

BY MARY ROWLEY-LAWSON, M.D.

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PLATES 51 TO 55.

In previous publications[1] I have stated that I believe the malarial parasite to be extracellular and to migrate from corpuscle to corpuscle. To recapitulate:

I believe the malarial parasite to be extracellular throughout its existence; that is, except for brief periods when it is free in the blood serum, it is attached to the external surface of the red corpuscle.

The parasite attaches itself to the red corpuscle by means of delicate pseudopodia thrown out from the cytoplasm of the parasite. It encircles and squeezes up into a mound a portion of the hemoglobin (figures 1 to 17); thus it maintains its position on the outside of the red corpuscle.

From my observations I believe that each parasite in the course of its development, destroys several red corpuscles, migrating from one to another, thus giving a rational explanation of the anemia which occurs in the course of the malarial infections.

The evidence in favor of migration is as follows: 1. The great destruction of red corpuscles, which is usually out of all proportion to the number of parasites present in the peripheral blood, provided that each parasite destroys but one corpuscle. 2. Instances of multiple infection of red corpuscles by several young parasites,

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[1] Rowley-Lawson, M., *Arch. Int. Med.*, 1912, ix, 420; *Jour. Exper. Med.*, 1913, xvii, 324.

sometimes as many as seven. They cannot all grow on one corpuscle, and even if conjugation were amongst the possibilities, it would not account for odd numbers nor for infection of a corpuscle by parasites of varying stages of development. Therefore if these parasites are not to die, they must migrate. 3. What appear to be stages in parasitic migrations, which may be summarized as follows: (a) Pigmented parasites free in the blood serum. They may be seen in various stages of development, compact, ring-shaped, ameboid, and with protoplasmic pseudopodia, the pigmentation being evidence of previous attachments to red corpuscles. (b) Pigmented parasites in various stages of development, attached to red corpuscles the hemoglobin of which is apparently unaltered. Here, as in stage (a), the pigmentation is evidence of one or more previous attachments. (c) Pigmented parasites in various stages of development on decolorized red corpuscles and on corpuscular skeletons. (d) Pigmented parasites partly on and partly off degenerated red corpuscles, caught apparently in the act of abandoning them. (e) Corpuscular skeletons, which are the expanded, dehemoglobinized remains of red corpuscles, usually more or less semilunar in shape, granular, and staining a delicate pink. These skeletons are most frequently seen free from parasites.

This paper is intended principally as a description of stage (d), but it illustrates also the extracellular relation of the adult tertian parasite to the red corpuscle. In one specimen of blood from a series of smears taken consecutively, one after another, from a patient who had taken no quinine, I found over 100 thirty (?) hour parasites partly on and partly off expanded red corpuscles showing granular degeneration (figures 19 to 54, and 59 to 78). I have occasionally seen parasites partly off red corpuscles in other specimens; but never so many in any one smear as in this case. Free parasites, in the same stage of development as the parasites seen partly off the red corpuscles, were found in the same smear (figure 55). I believe that these parasites were preparing to migrate, for the infected red corpuscles, though retaining in general their contour, were undoubtedly degenerated,[2] being more or less dehemoglobinized. Finding so many parasites in similar stages of development in this phase, in one smear, seems to me good evidence in favor of migration as against a condition caused by technique. I can think of no evidence in favor of technique that may be responsible for the phenomenon. The smear was made on a slide, with the blood spread only in one direction, yet the parasites were seen reaching away from the corpuscles in various directions. If rough smearing was the cause of the parasites being partly off the corpuscles, the parasites would be drawn off from the corpuscles all in one direction. And technique would not account for a condition where the corpuscle is distorted exactly at right angles to the direction in which the parasite reaches out from the corpuscle (figures 22 and 27).

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[2] Segmenting parasites on red corpuscles showing similar granular degeneration (figures 56 to 58) were found in the same smear.

In many instances that portion of the parasite which is off the red corpuscle, may be seen resting along the periphery of the infected cell (figures 20, 31, 32, 34 to 37, and 39), which would not be the case were technique responsible for its dislocation.

That the adult parasite is attached to the external surface of the red corpuscle and not submerged beneath its surface seems to me to be proved by figures 1 to 58.

Parasites developed beyond the young ring-form stage (figures 1 to 11) may occasionally be seen to be attached to fairly healthy appearing red corpuscles, the bodies of the parasites resting on the periphery of the corpuscles (figures 13 to 16). These parasites are certainly not submerged beneath the surface of the red corpuscles, but have, I believe, only recently attached themselves, and are therefore coming rather than going. Occasionally a segmenting body may be seen extending beyond the periphery of a red corpuscle with unbroken contour (figure 18).

As to the parasites that are preparing to migrate (figures 19 to 54), to the trained eye those that are on the upper surface of the granular degenerated corpuscles can easily be differentiated from the parasites on the under surface; i. e., in figure 22, the parasite is partly off the upper surface of the red corpuscle, while in figure 33 the parasite is seen to be partly off the under surface of the corpuscle.

The unbroken contour of the majority of the infected red corpuscles showing portions of parasites extending beyond the periphery, as well as appearance of the adjacent cells, with the exception of a in figure 36, would seem to preclude any suggestion of unusual violence in the spreading of the smear. I use the adjective unusual, as it seems to me that it would require a very unusual violence to produce a condition as rare as that seen in the accompanying plates. I believe the infected red corpuscles were already too much damaged by the action of the parasites to regain their contour on the slide if the parasites had been forcibly squeezed or pulled out of them in spreading the smear, especially parasites of such advanced growth. I do not believe that in smearing the blood it would ever be possible to reach the parasite, if it were submerged beneath the surface of the red corpuscle, in order to pull or squeeze it out, without damaging that corpuscle beyond repair; for instance, in figure 20 the body of the parasite rests on the periphery of the corpuscle and only short pseudopodia are seen to be attached to the corpuscle, the contour of which is unbroken; and in figure 19 only a slender process extends beyond the periphery of a corpuscle with unbroken contour. If these parasites were submerged beneath the surface of the red corpuscles, could technique have produced the condition? I think not.

Healthy appearing red corpuscles may be damaged by technique when the blood is being smeared and not regain their contour. The healthy, uninfected, but distorted, red corpuscle seen at a in figure 35 did not regain its shape after being injured by obvious technique. In malarial infections, especially in the æstivo-autumnal infections I have seen many red corpuscles which had not regained their normal contour after having been damaged by the parasites while in the circulating blood.[8]

If the young parasite fastens itself to the external surface of a red corpuscle and proceeds with the destruction of the corpuscle while it is so attached (see young parasites encircling corpuscular mounds on the decolorized red corpuscles in figures 10 and 11), why should the adult parasite follow any other procedure? Indeed, peripheral mounds may be seen in connection with adult parasites

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[8] An article describing and illustrating these damaged red corpuscles will appear later.

(figure 17, a). And why should the adult parasite assume a more or less characteristic ring-form unless it be for the purpose of securing its attachment to the surface of the red corpuscle? For the ring shape, in connection with the adult parasite, may be explained, as it is explained in connection with the young parasite, as a result of the parasite encircling and drawing up into a mound a portion of the hemoglobin substance of the red corpuscle in order to secure its attachment to the surface of the corpuscle.

In fresh blood preparations, parasites may be seen to abandon red corpuscles which have not been entirely destroyed. Laveran,[4] in referring to the external relation of the malarial parasite to the red corpuscle, says: "Osler noticed that the amoeboid bodies which adhere to the red blood corpuscles can be detached and become free in the blood," adding, that this was one of the arguments on which he relied in maintaining that the parasites were only attached to the surface of the red corpuscles. Marchiafava and Bignami[5] and Monacho and Panichi[6] have stated that quinine may cause certain parasites to abandon the red corpuscles. This being so, if the parasites were submerged beneath the surface of the red corpuscles, then the quinine would have to destroy the substance of the red corpuscles in order to release the parasites; but with the parasites attached to the external surface of the corpuscles, their detachment is more easily explained.

SUMMARY.

1. What appear to be certain definite stages in the migration of the malarial parasite from red corpuscle to red corpuscle may be demonstrated by thorough and persistent observations,—not minutes spent on each specimen, but many hours.

2. The migration of the malarial parasite from red corpuscle to red corpuscle gives a reasonable explanation of the loss of red corpuscles which cannot be accounted for by the destruction of the infected corpuscles at the time the parasites segment.

3. Migration to other red corpuscles is a satisfactory explana-

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[4] Laveran, A., Paludism, translated by Martin, J. W., London, 1893, 41.

[5] Marchiafava, E., and Bignami, A., in Twentieth Century Practice of Medicine, New York, 1900, xix, 461.

[6] Monacho and Panichi, cited by Ewing, J., Clinical Pathology of the Blood, 2d edition, New York and Philadelphia, 1903, 458.

tion of the ultimate fate of the young parasites seen in instances of multiple infection of single corpuscles.

4. In the light of the facts here presented, it would seem impossible to explain the instances of the parasites partly on and partly off degenerated red corpuscles as the result of technique. A stage in the migration of the parasite seems to me to be the probable interpretation of the phenomenon.

5. A corpuscular mound encircled by an adult parasite, when seen at the periphery of the red corpuscle, should have the same significance and interpretation in reference to the extracellular relation of the parasite to the corpuscle, that it has when it is seen in connection with a young parasite.

6. The adult ring-form parasite should have the same interpretation as the young ring-form parasite.

7. Attachment to the external surface of the red corpuscles seems to me to be the only possible interpretation of the appearances of the parasites pictured in this article.

EXPLANATION OF PLATES.

PLATE 51.

TERTIAN MALARIAL PARASITES.

Magnification, × 1,750.

FIGS. 1 to 3. Young parasites attached to the external surface of the red corpuscles. Those attached to peripheral mounds may be seen at a; the other parasites are attached to surface mounds.

FIG. 4. The nucleus of the young parasite attached to the peripheral corpuscular mound at a is distorted by technique.

FIG. 5. A young parasite attached to a peripheral corpuscular mound at a, which is almost entirely dehemoglobinized.

FIG. 6. A young parasite attached to the red corpuscle. The body of the parasite rests on the periphery of the corpuscle.

FIG. 7. A young parasite attached to the periphery of a peripheral corpuscular mound. The mound is almost entirely dehemoglobinized.

FIGS. 8 and 9. Young parasites encircling peripheral corpuscular mounds.

FIG. 10. A young parasite encircling a surface corpuscular mound; the entire corpuscle is much dehemoglobinized.

FIG. 11. A young parasite encircling a peripheral corpuscular mound. The corpuscle is much dehemoglobinized.

FIG. 12. A pigmented parasite attached to the surface of a red corpuscle. The mounds to which it is attached can be seen at a.

FIG. 13. A pigmented parasite attached to the surface of a red corpuscle, the hemoglobin of which appears to be unaltered. As a large part of the parasite

rests on the periphery of this corpuscle, I believe that the parasite has only recently attached itself to it, hence the unaltered appearance of the hemoglobin.

Fig. 14. A parasite attached to the external surface of the corpuscle. The body of the parasite is seen to be off the corpuscle, having been dislocated by technique.

Fig. 15. A pigmented parasite on the periphery of the red corpuscle, the appearance of the pseudopodia suggesting that the parasite is starting to encircle a corpuscular mound. The slightly degenerated appearance of the infected corpuscle is probably due to the action of the young parasite.

Fig. 16. A pigmented parasite, the body of which rests on the periphery of the corpuscle. This parasite is attached to the under surface of the red corpuscle. There is another parasite attached to the upper surface.

Fig. 17. An adult pigmented parasite attached to the external surface of the red corpuscle. The peripheral mound to which it is attached can be seen at a.

Fig. 18. Young parasites resulting from a very recent segmentation; the red corpuscle has not been entirely destroyed. One of the segments can be seen to be partly off, and two entirely off the corpuscle at a.

Fig. 19. An adult pigmented parasite on the upper surface of a granular degenerated red corpuscle. A pigmented protoplasmic process arising from the parasite can be seen extending beyond the periphery of the corpuscle at a.

Fig. 20. An adult parasite resting on the periphery of a granular degenerated red corpuscle. With the exception of three short and delicate pseudopodia, the parasite is free from attachment to the corpuscle.

Fig. 21. An adult parasite on the upper surface of a degenerated red corpuscle, with a portion of its protoplasm extending beyond the periphery of the infected corpuscle.

Plate 52.

TERTIAN MALARIAL PARASITES.

Magnification, × 1,750.

Fig. 22. An adult parasite partly on and partly off the upper surface of a degenerated red corpuscle. Note that the parasite is lying off the corpuscle at right angles to the way the cell is distorted.

Fig. 23. An adult parasite partly on and partly off the upper surface of a degenerated red corpuscle. The mound to which the parasite is attached shows less of the granular degeneration than the rest of the corpuscle.

Fig. 24. An adult parasite partly on and partly off the upper surface of a degenerated red corpuscle. The surface mound to which the parasite is attached is almost entirely decolorized by the action of the parasite. This mound can be seen at a.

Figs. 25 and 26. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. The mound to which the parasite in figure 26 is attached can be seen at a.

Fig. 27. An adult parasite partly off the under surface of a degenerated red corpuscle. The granular outline of the infected corpuscle can be traced across the upper surface of the parasite between o and o. The mound to which the parasite is attached can be seen at a. Note that the distortion of the corpuscle is at right angles to the way the parasite lies off the corpuscle.

FIGS. 28, 29, and 30. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. The mounds of attachment of the parasite in figure 30 can be seen at a.

FIGS. 31, 32, and 33. Adult parasites partly on and partly off the under surfaces of degenerated red corpuscles. In figures 31 and 32, the part of the parasite that is off the corpuscle can be seen resting on the periphery of the infected corpuscles.

PLATE 53.

TERTIAN MALARIAL PARASITES.

Magnification, × 1,750.

FIG. 34. An adult parasite partly on and partly off a degenerated red corpuscle; the part of the parasite that is off the corpuscle may be seen resting on the periphery of the corpuscle.

FIG. 35. An adult parasite partly on and partly off the upper surface of a red corpuscle which is less dehemoglobinized than the other corpuscles shown which are infected by parasites partly off and partly on. At a is a red corpuscle that has been injured by technique and has not regained its normal shape.

FIGS. 36 to 39. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. In figures 36, 37, and 39 the part of the parasite which is off the corpuscles can be seen resting on the periphery of the infected corpuscles. In figure 37 a young parasite is seen attached to the infected corpuscle.

FIG. 40. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle. Another adult parasite can be seen on the under surface of the infected corpuscle.

FIGS. 41 and 42. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles.

FIG. 43. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle. The mound of attachment is seen at a.

FIGS. 44 and 45. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles. In figure 44 a young parasite is seen attached to the infected corpuscle. The mound to which the adult parasite is attached can be seen at a.

PLATE 54.

TERTIAN MALARIAL PARASITES.

Magnification, × 1,750.

FIG. 46. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle.

FIGS. 47, 48, and 49. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles.

FIG. 50. An adult parasite partly on and partly off the under surface of a degenerated red corpuscle.

FIGS. 51, 52, 53, and 54. Adult parasites partly on and partly off the upper surfaces of degenerated red corpuscles.

FIG. 55. An example of the free parasites found in the same smear with, and in the same stage of development as, the parasites seen partly on and partly off the degenerated red corpuscles.

FIGS. 56, 57, and 58. Segmenting parasites on corpuscles which are in a similar stage of degeneration to many of the corpuscles which show the parasites partly off them, and which came from the same smear.

PLATE 55.

TERTIAN MALARIAL PARASITES.

Magnification, × 1,750.

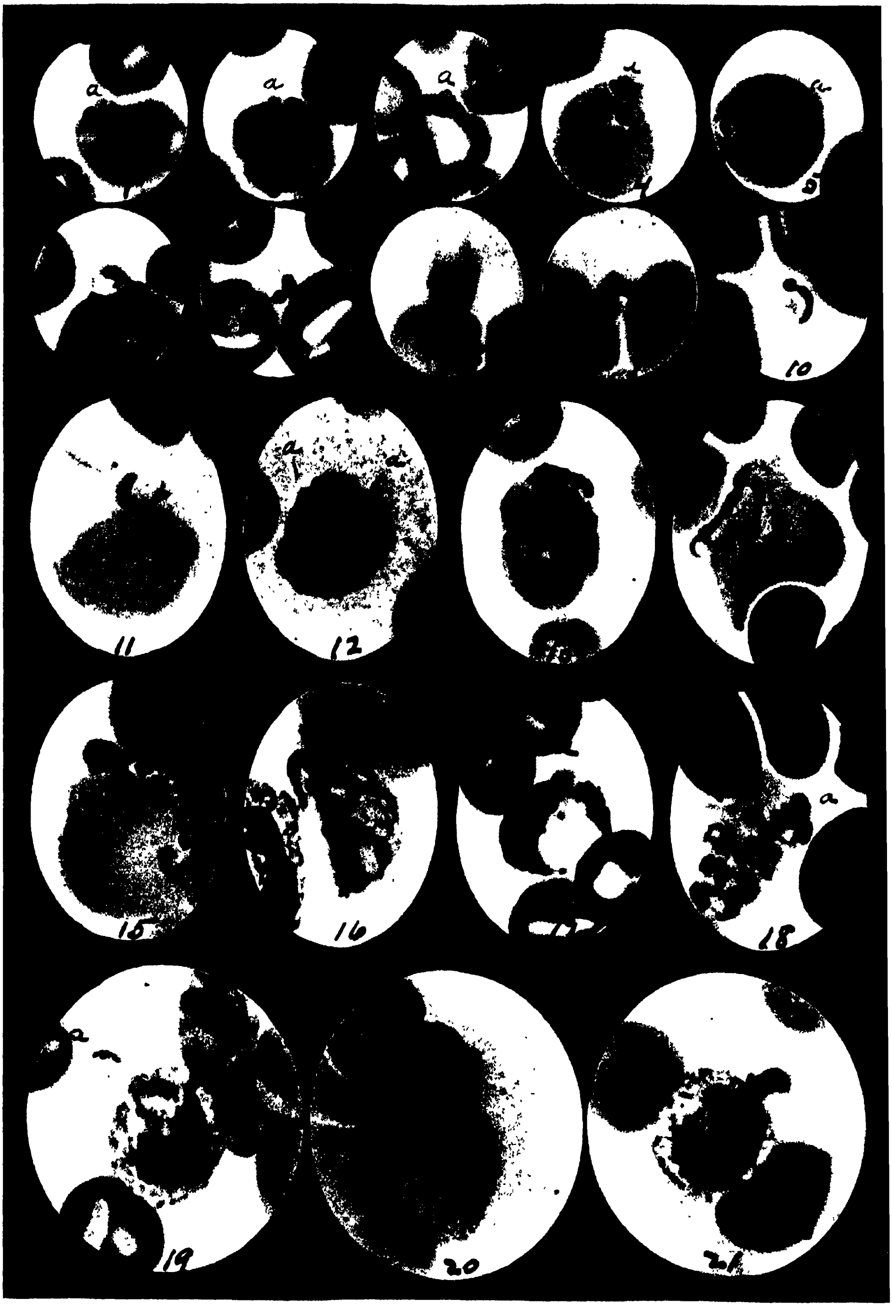
FIG. 59. (Corresponds to figure 18.) A segmenting parasite showing some of the separate segments beyond the periphery of the infected red corpuscle.

FIG. 60. Two young parasites attached to the surface of a red corpuscle; the body of one of the parasites is on the periphery of the corpuscle with the attaching pseudopodium arranged in the form of a loop overlying the corpuscular substance; the other parasite is attached to a decolorized surface mound.

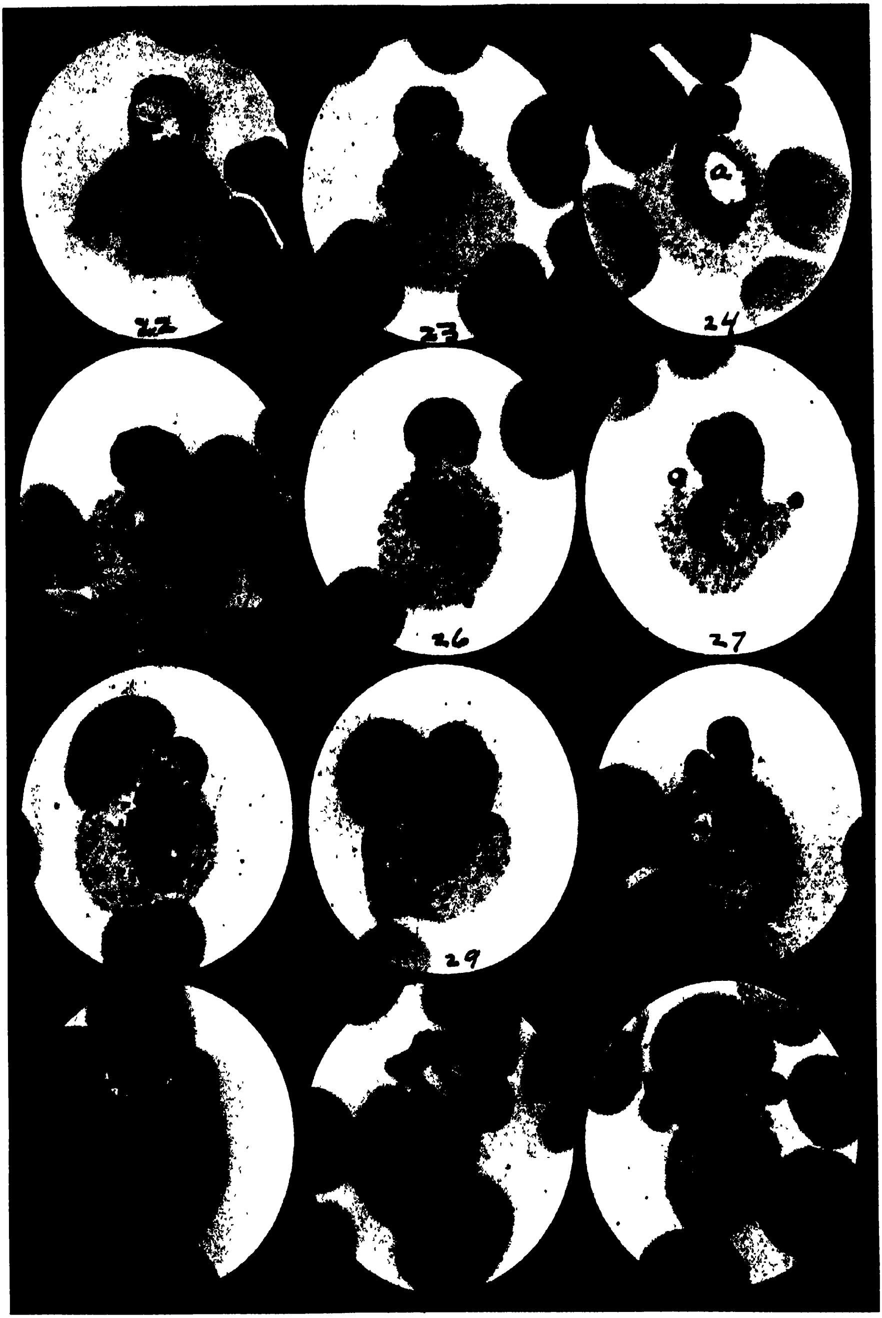
FIG. 61. (Corresponds to figure 12.) A pigmented parasite attached to the surface of a red corpuscle. The mounds to which the parasite is attached can be seen.

FIG. 62. (Corresponds to figure 15.) A pigmented parasite on the periphery of a red corpuscle, with the pseudopodia overlying the corpuscular substance.

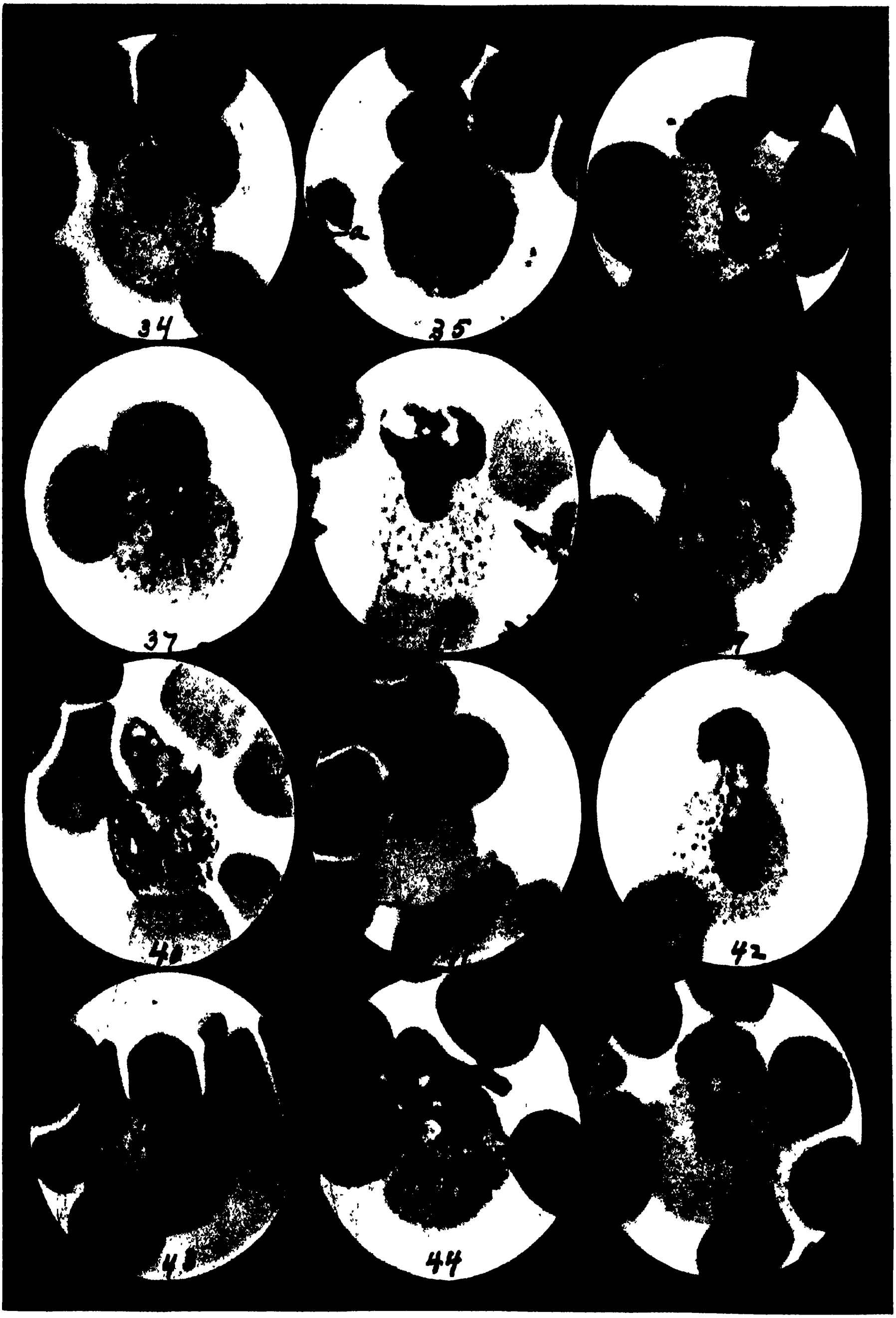
FIGS. 63 to 78. (Correspond to figures 25, 28, 23, 24, 37, 26, 30, 22, 29, 33, 32, 31, 21, 20, 19, and 36.) Adult pigmented parasites partly on and partly off granular degenerated red corpuscles. Some of the parasites may be seen to be on the upper surfaces of the corpuscles, others on the under surfaces. In figure 18, with the exception of three short and delicate pseudopodia, the parasite is free from attachment to the corpuscle. In figure 19 only a delicate pseudopodium extends beyond the periphery of a red corpuscle with unbroken contour.



(Lawson: Migration of the Adult Tertian Malarial Parasite.)



(Lawson: Migration of the Adult Tertian Malarial Parasite.)



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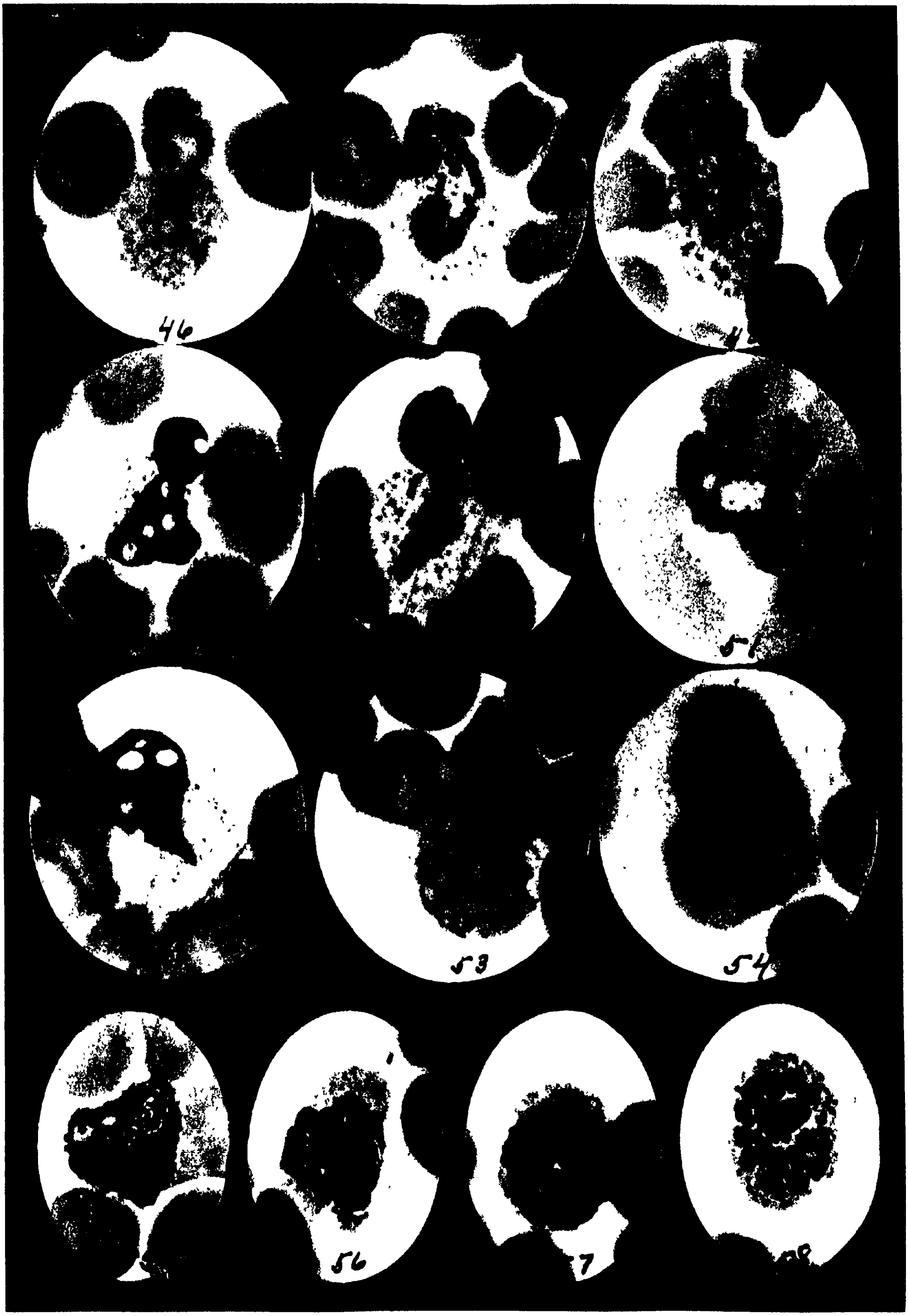
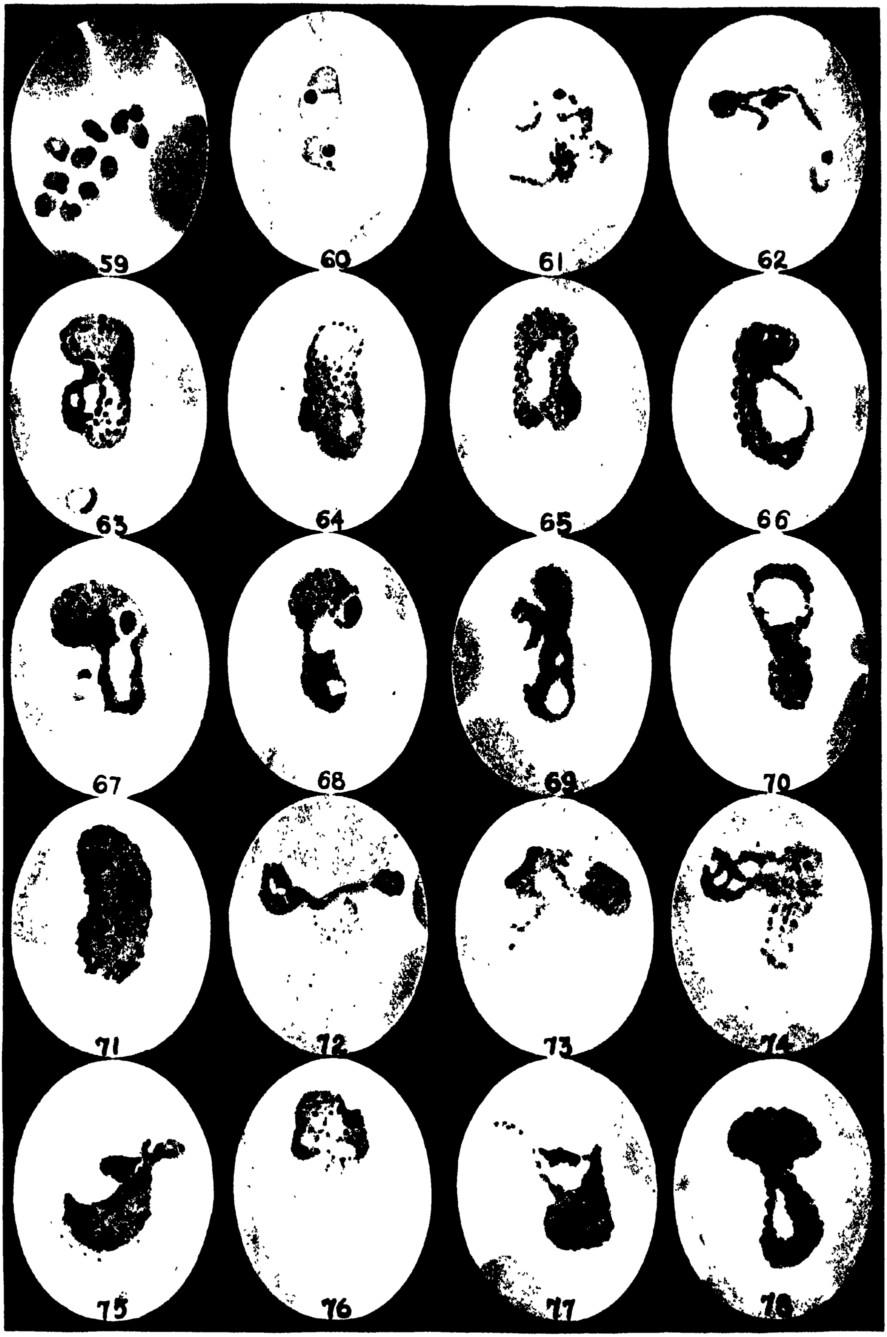


(Lawson: Migration of the Adult Tertian Malarial Parasite.)



(Lawson: Migration of the Adult Tertian Malarial Parasite.)

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FACTORS OF RESISTANCE TO HETEROPLASTIC TISSUE-GRAFTING.

Studies in Tissue Specificity. III.*

By JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Plates 56 to 60.

Previous observations have tended to show conclusively that tissues cannot be transplanted from one species to another, even though these be closely related. Two theories have been brought forward to explain this failure in heteroplastic grafting. The two schools are still at variance and neither has been able to produce evidence conclusive enough to convince the other. The first and most prominent theory is that of Ehrlich, termed athrepsia.[1] The experimental foundation for this hypothesis is the so called zigzag transplantation of tumors between rats and mice. It was observed that a mouse tumor when grafted into a rat, or *vice versa,* would survive and proliferate for six to eight days, but would later fail rapidly and be absorbed. If, however, the mouse tumor was removed during the proliferating stage and reinoculated into a mouse it continued to grow actively. After a period of six or eight days' active growth in the mouse it could again be grafted into a rat. This zigzag grafting could be carried on indefinitely with no apparent effect on the tumor tissue or in lessening its activity of growth. The interpretation, suggested by Ehrlich, is that each species provides its tissues with a specific food substance (X) which is necessary for its maintenance and growth. The temporary survival of the mouse tissue in the rat is due to the amount of this specific food carried over with the graft. When this is exhausted the graft dies unless returned to its native species, where it will accumulate a fresh supply of the specific food and again be able to survive for a time in a foreign species.

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* Received for publication, March 24, 1914.

[1] Ehrlich, P., *Arb. a. d. k. Inst. f. exper. Therap.*, 1906, No. 1, 84.

The chief opponent of this theory is Bashford[3] who rests his objection on the findings in an experiment in which rats were inoculated a second time with mouse tumor. Under these conditions the second graft, although containing an equal amount of the hypothetical food substance, would survive only two to three days. From this fact he concludes that there is an active immunity developed against the cancer cell as a foreign proteid. The time of survival of the first graft he considers the time required for the development of the active immunity. Bashford[3] claims that the immunity to homoplastic grafting is an entirely different process and that it depends entirely on the blood vessel and stroma reactions. The merits of the two theories will not be discussed; they are quoted to give an idea of the present views on the subject.

LYMPHOCYTIC REACTION IN RELATION TO TISSUE GRAFTS IN IMMUNE ANIMALS.

The occurrence of a lymphocytic reaction around tissue grafts in immune animals has been pointed out by numerous observers[4] and arises whatever the type or source of the animal's immunity. The immune states are: the natural immunity possessed by an animal individually, or because of variety of species; the acquired immunity which is present in animals that have recovered from a primary or implanted tumor; and finally the so called artificial immunity which can be induced by one of several procedures. The small round cell or lymphocytic infiltration is present when there is healing in a spontaneous tumor, and is seen around the edge of slowly growing cancers in man. The importance of these cells in the immunity reaction to tissue grafts would seem evident, yet they have received scant attention.

HETEROPLASTIC GRAFTING IN A NON-RESISTANT ORGANISM, THE CHICK EMBRYO.

In a previous communication[5] it has been pointed out that the avian embryo has no defensive mechanism against the growth of

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[2] Russell, B. R. G., *Third Scientific Report of the Imperial Cancer Research Fund*, 1908, 341.

[3] Bashford, E. F., and Russell, B., *Lancet*, 1910, i, 782.

[4] For the literature see Da Fano, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, v, 1.

[5] Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1912, lix, 874.

tissues of a foreign species. The tumor tissue of a rat, for instance, by transference from embryo to embryo could be kept growing in the chick for an indefinite period.[6] The rat tissue underwent no marked change during its long sojourn in the chick embryo, as was shown by the fact that at any time during this period it could be replanted successfully into its native species but was promptly disintegrated when grafted into the adult chicken. It was later shown[7] that a defensive mechanism developed rapidly in the embryo at about the time of hatching, quickly destroying any foreign tissue that might be present. The foreign species tissue growing in the embryo shows a total absence of a round cell reaction. The lymphoid cells around the graft first become evident at about the time that the defensive mechanism begins to show its effect. Other than this there is no great change in the embryo to account for this sudden development from a susceptible to a highly resistant organism. If this change is the result of the sudden activity of a tissue or organ formerly quiescent, it should be possible to provide the embryo with this necessary tissue or organ by grafting various adult tissues into the chick embryo.[8]

ACTION, IN VITRO, OF TISSUES IN HOMOLOGOUS PLASMA ON THE GROWTH OF HETEROLOGOUS TISSUE.

Lambert and Hanes[9] have shown that the tissues of one species are capable of growth in the plasma of certain other species. As a preliminary step to the experiment suggested above an attempt was first made to determine the interaction of tissues *in vitro.* Bits of a rapidly growing rat sarcoma were placed in drops of chicken plasma and to these were added in series bits of various adult chicken tissues. The cultures were mounted in hollow slides according to the well known method. The rat tissue in chicken plasma grew remarkably well and was not affected by adult chicken connective tissue, kidney, or liver in close proximity. When, however, a bit of adult chicken spleen was growing in the same drop of plasma there was practically a total inhibition of the growth of the rat sarcoma. The only other tissue showing a similar effect was the bone marrow, which caused definite retardation, but not so marked an inhibition as that brought about by the spleen.

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[6] Murphy, Jas. B., *Jour. Exper. Med.,* 1913, xvii, 482.

[7] Murphy, Jas. B., *idem,* 1914, xix, 181.

[8] Murphy, Jas. B., *Jour. Am. Med. Assn.,* 1914, lxii, 199.

[9] Lambert, R. A., and Hanes, F. M., *Jour. Exper. Med.,* 1911, xiv, 129.

THE EFFECT OF ADULT CHICKEN TISSUE GRAFTS ON THE HETEROPLASTIC GRAFTING IN THE EMBRYO.

Since it is possible to graft various adult tissues into the embryo[10] the above experiment was repeated *in vivo*.

In the first series, comprising twenty experiments and over 150 embryos, grafts of rat sarcoma and bits of adult chicken tissues were placed side by side in the outer membrane of seven day chick embryos, according to the method previously described. The adult chicken tissues used were spleen, kidney, liver, bone marrow, and connective tissue. The eggs were returned to the incubator, and at intervals up to the eighteenth day of incubation part of each lot was opened and the grafts were removed for microscopic examination. Text-figure 1 shows the results in a few of these experiments. In the instances where adult chicken kidney and rat sarcoma were inoculated together the resultant tumors were as large as the controls of rat tumor alone; that is, they generally measured from one to two centimeters. Microscopic examination showed the rat cells in active proliferation, with the kidney tubules, also in good condition, scattered through the tumor mass or in a clump at its edge (figure 1). Chicken liver grafts generally caused a widespread necrosis of the membranes of the chick. When bits of the liver graft survived they were found to consist of a few scattered bile capillaries. If the rat tissue graft escaped the necrosis it was found to be in as active growth as the controls. Connective tissue of the adult chicken had no effect on the rat tumor cells in the embryo, although the connective tissue itself grew well.

The striking result was obtained when grafts of adult chicken spleen were inoculated with the rat tumor. The resulting tumors instead of being well rounded, greyish, and semitranslucent were flat, often mottled, yellowish, and opaque. Microscopic examination of specimens removed after three or four days showed the

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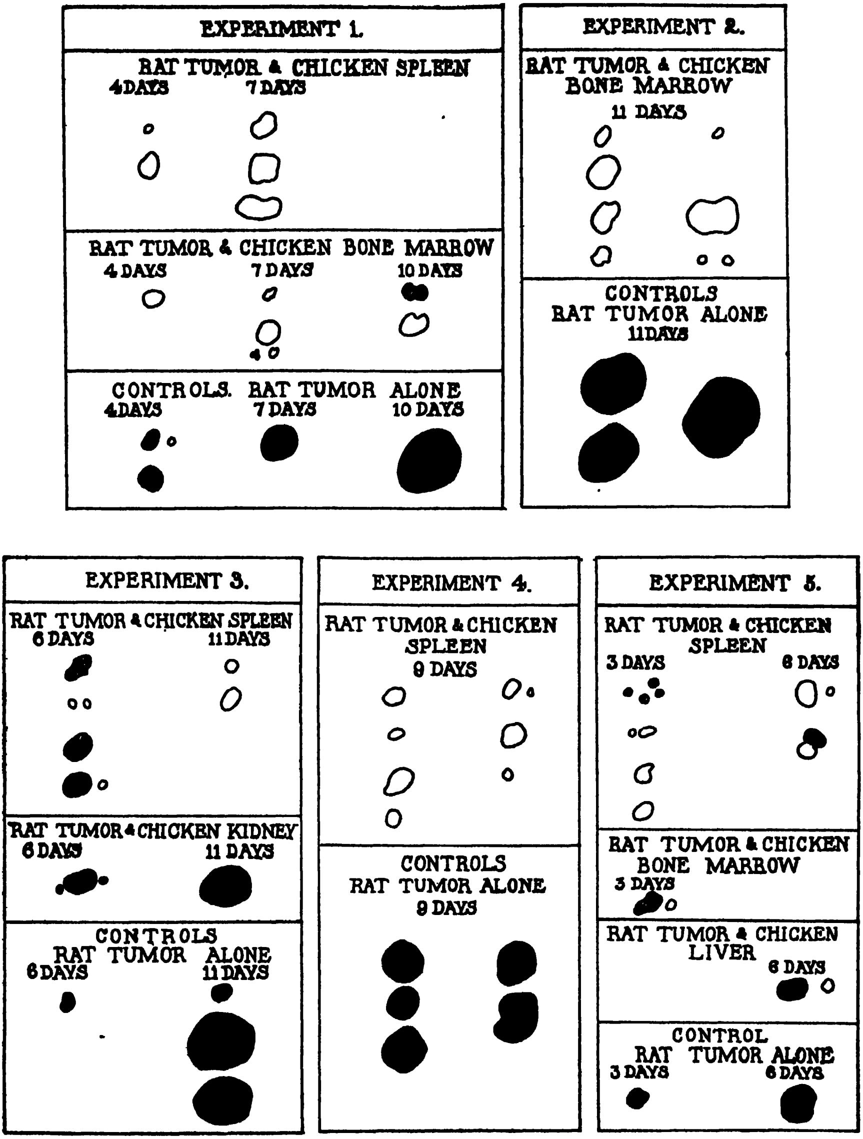
[10] Murphy, Jas. B., *Jour. Exper. Med.*, 1913, xvii, 482.



Text-Fig. 1. This chart shows in silhouette the results of simultaneous inoculation of rat sarcoma and a graft of adult chicken tissue into the outer membrane of chick embryos. The unshaded nodules were found on microscopic examination to be made up of the adult chicken tissue and reactive tissue, but showed no surviving rat cells. The shaded nodules were found to have a few rat cells embedded in a mass of reactive tissue (figures 2, 3, and 6). The black represents tumors in active growth, with no sign of defensive reaction.

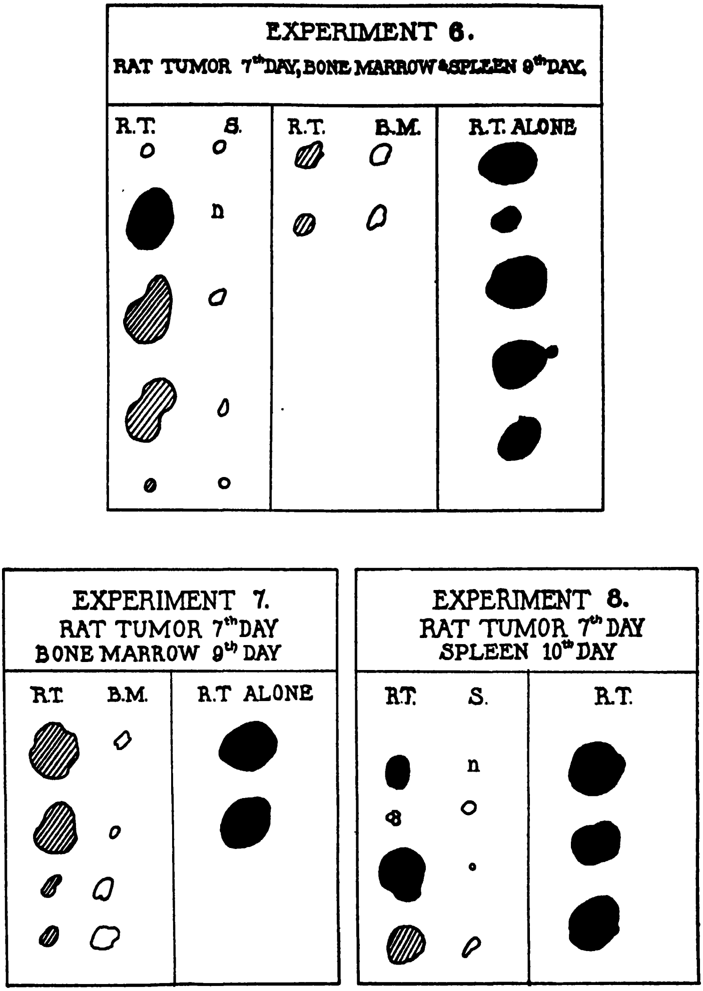
spleen graft well established, made up of the typical spleen cells. The rat tissue was found much degenerated, surrounded by collections of small round cells and largely replaced by connective tissue (figure 2). Later stages show the rat tissue to be dead, embedded in a mass of connective tissue with clumps of small round cells scattered throughout the surrounding tissue (figure 3). This condition offers a strong contrast to the picture shown by the controls of the rat tumor inoculated alone. Here the rat cells are practically devoid of stroma and the edges show no reactive tissue (figure 4). The final stages of rat tumor and chicken spleen showed the spleen graft to be in good condition, but with no evidence of the rat tissue remaining (figure 5).

The action of bone marrow resembled that of the spleen (figure 6), but was slower and less complete. The bone marrow grafts were composed for the most part of fat cells and collections of lymphoid cells.

THE EFFECT OF CHICKEN SPLEEN AND BONE MARROW ON ESTABLISHED GRAFTS OF FOREIGN TISSUES IN THE EMBRYO.

The fact that the tissues in the foregoing experiments were growing side by side and often intermingling presents a difficulty in the interpretation. The present series of experiments was planned to avoid the contact action and to give the spleen and bone marrow a more severe test. The kidney (figure 7) and other tissues mentioned above have no evident effect even in contact and they were therefore not used in this experiment.

Series of eggs were inoculated with rat sarcoma in the usual way on the seventh day of incubation. Two or more days later an opening was made on the opposite side of the eggs, and an adult chicken spleen or bone marrow graft was placed in the outer membrane. Some of the results are shown in outline in text-figure 2. On examination of these specimens eleven days later, the controls inoculated with rat tumor alone showed, almost without exception, large, well rounded, semitranslucent masses at the point of inoculation. In the embryos carrying a graft of adult spleen or bone marrow the tumors were flat, yellowish, and opaque. In some of these only a flake of tumor survived (figure 8). Microscopic ex-



TEXT-FIG. 2. This chart shows in silhouette the effect of adult chicken spleen and bone marrow on established and growing rat tumor in the embryo, when the adult tissues were inoculated at a distance. In the column with double rows of silhouettes the one on the left is the rat tumor (R.T.) and that on the right the bone marrow (B.M.) or spleen (S.) in the same embryo. The last column gives the controls of rat tumor alone. The day of incubation at which the inoculation was made is given in the caption. All tumors were removed at the eighteenth day of incubation. Black indicates that the tumors are composed of rat cells in active proliferation; the shaded outlines, that the rat tissue is much degenerated, with pronounced infiltration with round cells (figures 9 and 10). The unshaded outlines indicate that none of the rat cells survived. N indicates that graft did not take.

amination of the tumors showed massive collections of lymphocytes around the edges and in clumps associated with the blood vessels throughout the tumor (figures 9 and 10). There was a great increase in the connective tissue surrounding and replacing the rat tissue. The rat cells showed many degenerated forms and mitotic figures were rare. It would seem therefore that the adult spleen and bone marrow are capable of supplying a defensive mechanism to the chick embryo, even though the graft of spleen or bone marrow be some distance from the foreign tissue and introduced after the foreign tissue is established and actively growing.

DISCUSSION.

I shall make no attempt to discuss these findings in relation to the theories already brought forward to explain the failure of heteroplastic tumor grafts. The constant result obtained in a score of experiments or more shows conclusively that the adult spleen and bone marrow are capable of supplying a defensive mechanism to the chick embryo, which under ordinary conditions offers no resistance to the growth of foreign species tissue. Furthermore, the embryo bearing such grafts of spleen and bone marrow defends itself in the same way as the adult, if we may judge from the histological picture. The cells common to the graft of bone marrow and spleen, to the reaction around the foreign species graft in the adult, and to the embryo supplied with a defensive mechanism is the small lymphoid cell. It is therefore natural to suppose that this is an active agent in the defense.

Whether or not these lymphoid cells are the important factors in the failure of homoplastic grafts under certain conditions remains to be seen. Certainly a large preponderance of the evidence points in this direction. They are present often in large numbers around grafts of transplantable cancer in immune animals of the same species, regardless of the type of immunity. A recent communication of Apolant's[11] adds weight to this idea. He has shown that in splenectomized animals only a slight or very transient immunity can be developed to transplantable tumors. Oser and Pribram[12] have shown also that transplantable tumors grow more rapidly in splenectomized animals. The results reported by Baeslack[18] indicate that the number of small lymphocytes in the circulating blood of a tumor-bearing animal has a definite relation to the rate of growth of the tumor, falling rapidly in a susceptible animal and increasing steadily in an animal showing resistance.

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[11] Apolant, H., *Ztschr. f. Immunitätsforsch., Orig.*, 1913, xvii, 219.

[12] Oser, E. G., and Pribram, E. E., *Ztschr. f. exper. Path. u. Therap.*, 1912, ii, 295.

SUMMARY.

It has been shown that the chick embryo offers suitable conditions for the growth of implanted tissues, whether these be embryonic or adult, of the same species or a foreign one. The chick at about the time of hatching develops a defensive mechanism against the tissue of foreign species. This resistance can be supplied to the embryo in the early stages if grafts of adult spleen or bone marrow are implanted. Under these conditions the embryo exhibits the same resistance to foreign tissue as does the adult, and presents the same histological manifestations about the graft. Furthermore, the same tissues, spleen and bone marrow, when grafted into an embryo with an established and growing rat tumor, bring about a retrogression and absorption of the foreign tissue. Other adult tissues do not supply this power to the embryo.

EXPLANATION OF PLATES.

PLATE 56.

FIG. 1. The edge of a tumor resulting from a ten days' growth of a Jensen rat sarcoma and adult chicken kidney inoculated into the outer membrane of a seven day embryo. The kidney tubules are seen scattered around the edge of the tumor mass, which is made up of the rapidly grown rat cells.

FIG. 2. A section of a tumor resulting from a simultaneous inoculation of adult chicken spleen and a rat sarcoma, after five days' growth in the outer membrane of a chick embryo. A = spleen graft. B = the sarcoma cells surrounded and largely replaced by small round cells and connective tissue.

PLATE 57.

FIG. 3. The remains of the rat sarcoma cells eight days after inoculation into an embryo which at the same time received a graft of adult chicken spleen. The cell structure of the rat tissue is entirely lost and the whole is embedded in a thick connective tissue mass. Small round cells are seen scattered through the section. Compare with figure 4.

[18] Baeslack, F. W., *Ztschr. f. Immunitätsforsch., Orig.*, 1914, xx, 421.

FIG. 4. A section of a tumor resulting from an inoculation of the rat sarcoma alone, after eight days' growth in the chick embryo. Several mitotic figures are seen.

PLATE 58.

FIG. 5. The resulting tumor from a simultaneous inoculation of a seven day embryo with a rat sarcoma and a graft of adult chicken spleen, after eleven days. The spleen graft is seen on the left and the location of the sarcoma graft is on the right. There are no evidences of the rat cells remaining.

FIG. 6. This section shows the effect of a chicken bone marrow graft on a rat sarcoma after six days in the embryo. The rat cells (A) are embedded in a mass of small round cells (B).

PLATE 59.

FIG. 7. A drawing, somewhat enlarged, showing a tumor in the outer membrane of an eighteen day old embryo, resulting from a simultaneous inoculation eleven days previously of grafts of rat sarcoma and adult chicken kidney. The kidney is shown as the bluish nodule in the concavity of the tumor. The controls of rat tumor alone ranged about the same size.

FIG. 8. A drawing, somewhat enlarged, showing the effect of adult chicken spleen on an established graft of rat tumor in a chick embryo. The lower figure is the control of rat tumor alone after eleven days of growth in the outer membrane of chick embryo. The upper figure is the outer membrane of an embryo inoculated at the same time as the above with rat tumor (yellowish area), but two days later a graft of adult chicken spleen was added (pink nodule).

PLATE 60.

FIG. 9. Section of rat tumor in a chick embryo which had at some distance away a graft of adult bone marrow. A = round cell infiltration. B = degenerated rat cells. Compare with figure 4.

FIG. 10. Section of rat tumor in a chick embryo which had at some distance away a graft of adult chicken spleen. A = dense round cell infiltration at edge. B = degenerated rat cells lying in a mass of connective tissue. Compare with figure 4.

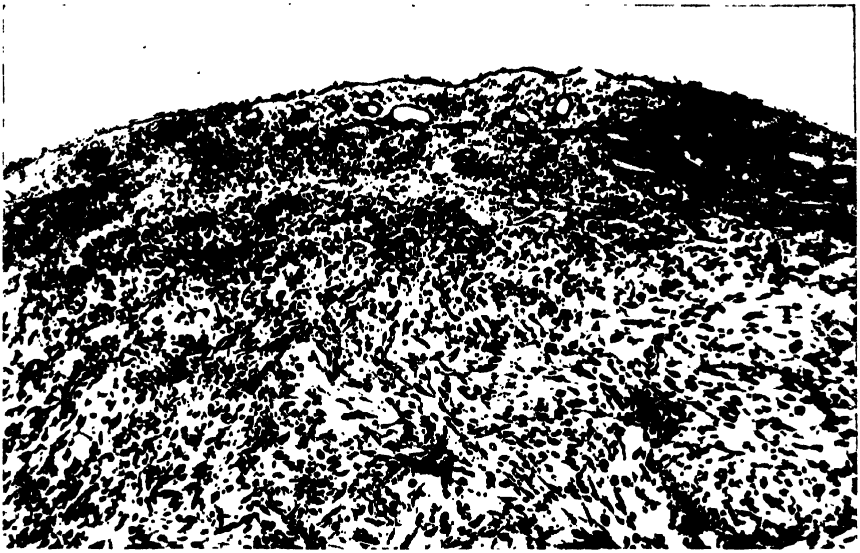


FIG. 1.

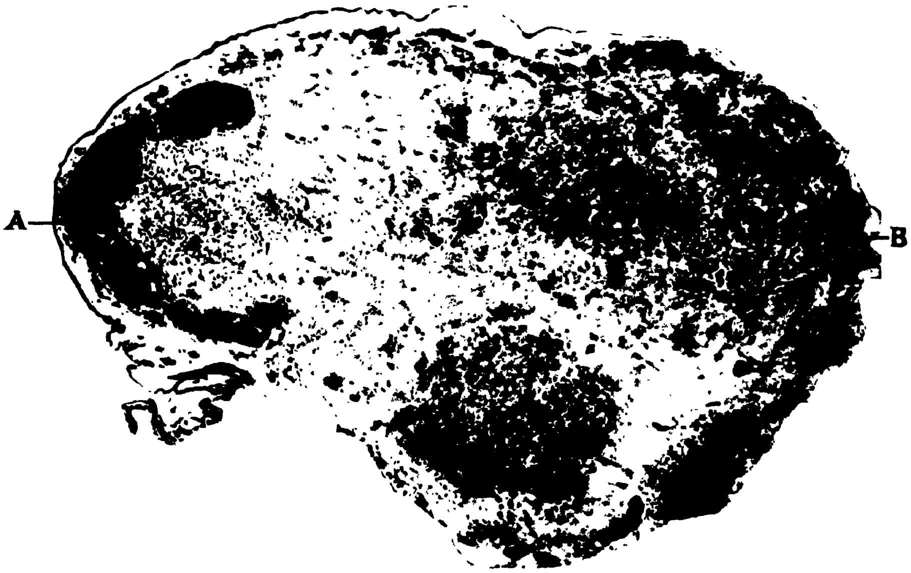


FIG. 2.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)

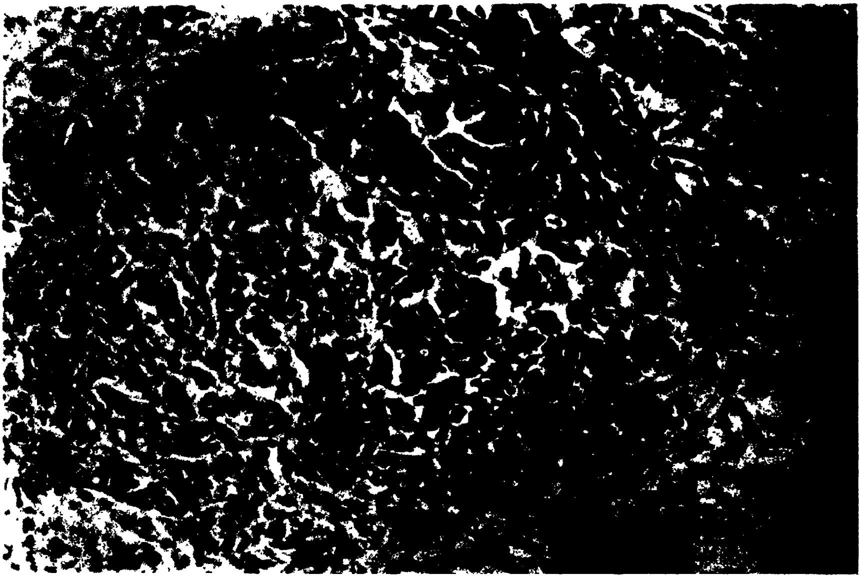


FIG. 3.

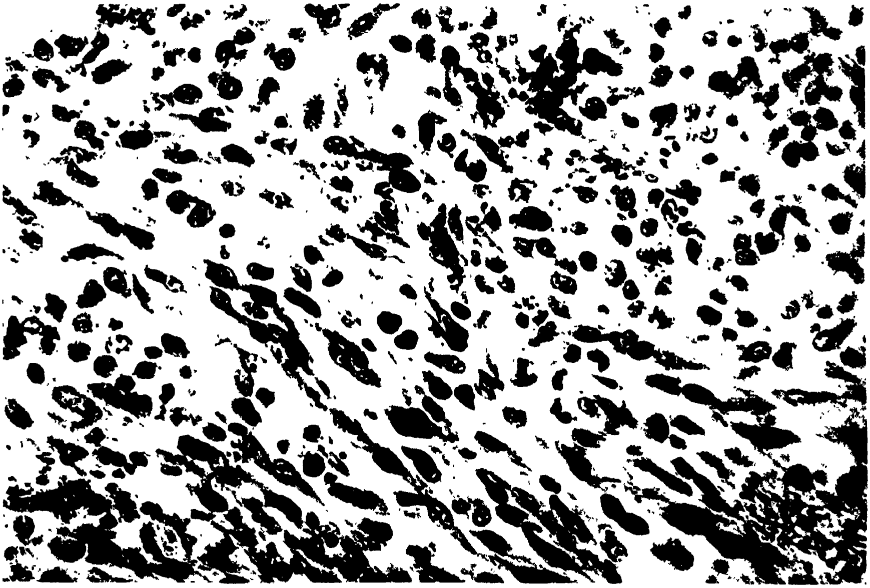


FIG. 4.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)

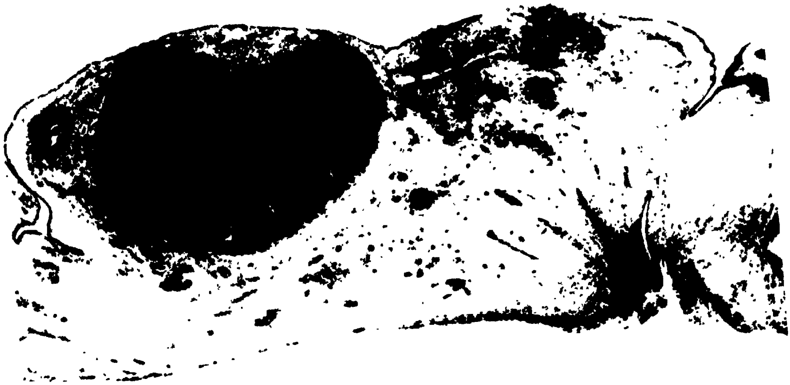


FIG. 5.

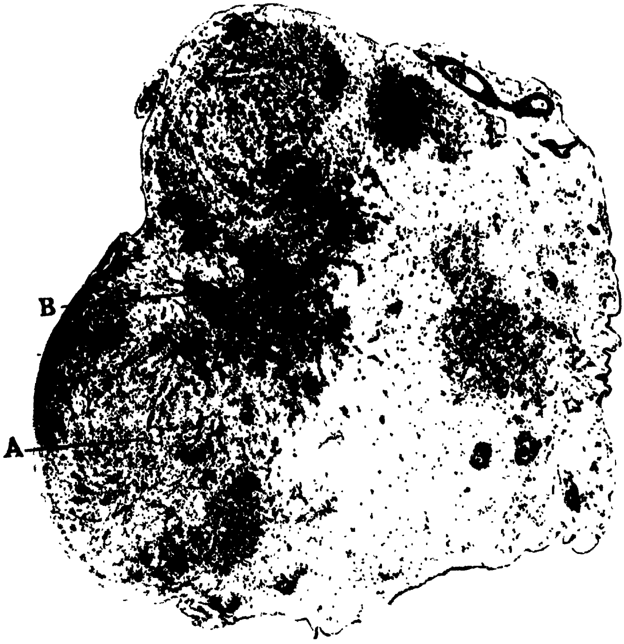


FIG. 6.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)

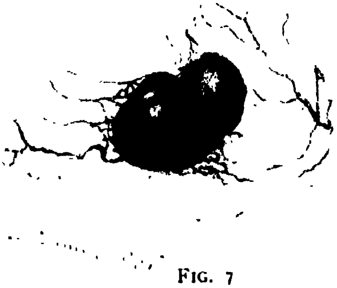
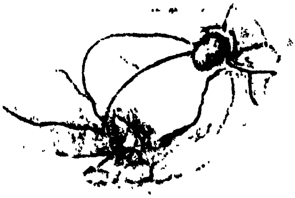


FIG. 7



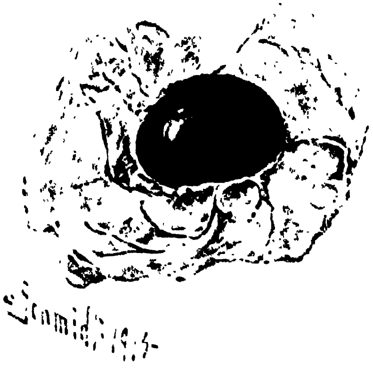
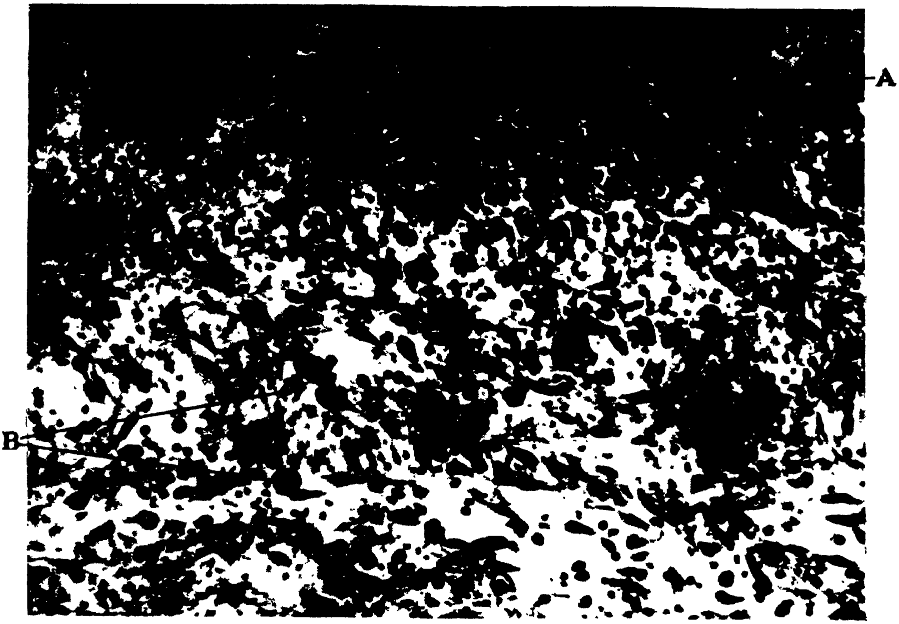


FIG. 8.

(Murphy: Factors of Resistance to Heteroplastic Tissue-Grafting.)



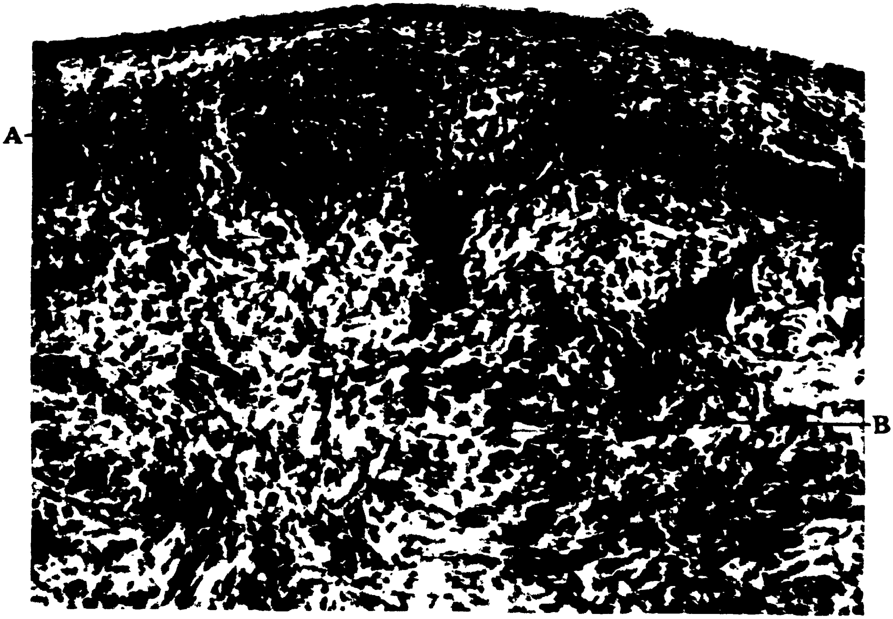


FIG. 10.

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FREE MALARIAL PARASITES AND THE EFFECT OF THE MIGRATION OF THE PARASITES OF TERTIAN MALARIAL INFECTIONS.*

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PLATES 61 TO 66.

I have shown in a previous paper[1] that the malarial parasite is extracellular throughout its life cycle; that in the course of its evolution, it may destroy a number of red corpuscles, migrating from one to another; and that in the intervals between its abandonment of the degenerated remnants of the red corpuscles (corpuscular skeletons) and its subsequent attachment to the surface of fresh red corpuscles, the parasite is, for very brief periods of time, free in the blood serum. The destruction of several red corpuscles by each parasite would account for the rapidity and severity of the anemia occurring in malarial infections.[2]

The free tertian parasites pictured in this article (with the exception of figures 96 to 99, 109 to 112, 114 to 116, 121, 122, and 124) came from two cover-slip smears; one of the specimens is from a series of smears taken at intervals of approximately one half minute, the other from a series of smears taken at intervals of one half hour. No quinine had been given.

In order to make clear the relation of the free parasites to migration, I shall describe briefly the process of migration.

Various Stages in the Migration of the Tertian Malarial Parasite.—(1) Pigmented parasites on decolorized red corpuscles or on corpuscular skeletons (granular degenerated remnants of red corpus-

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[1] Rowley-Lawson, M., *Arch. Int. Med.*, 1912, ix, 420.

[2] Marchiafava and Bignami (1) write: "In no other infection is anaemia produced with the same rapidity and to the same extent as in malaria."

cles); (2) pigmented parasites in the act of abandoning the decolorized or granular degenerated red corpuscles; (3) pigmented parasites, free in the blood serum; (4) pigmented parasites in the same stages of development as the parasites described under stages 1, 2, and 3, but attached to red corpuscles, the hemoglobin of which appears to be as yet unaltered.

The Rapidity with Which Migration May Take Place.—I believe the process of migration to be very rapid. The young unpigmented parasites usually appear almost immediately attached to red corpuscles. Thayer (2), in writing of these fresh segments, states: "They appear in the red corpuscles simultaneously with, or shortly after the appearance of the sporulating bodies." I believe that the free pigmented parasites in migration also attach themselves almost immediately to fresh red corpuscles. This belief is supported by the following facts: (a) all the above stages in migration may be present in one smear; and (b) only two successive smears, from the series of 200 smears taken at one half minute intervals, showed what I interpret as stages in migration.

Parasites Free in the Blood Serum.—At times there may be seen free in the blood serum segments resulting from a recent segmentation, and pigmented parasites in several stages of development and in various phases: (a) presegmenting (figures 110 and 111); (b) segmenting (figure 109); (c) individual segments (figures 36 and 96); (d) ring-form (figures 1 to 14, 48, 57, and 67); (e) ameboid (figures 62 to 64, 69, and 70); (f) compact (figures 16 to 35, 38 to 40, 47, 49, 51, 114 to 120, and 126); (g) with protoplasmic pseudopodia (figures 37, 41 to 46, 50, 52 to 56, 58 to 61, 65, 66, 68, 71 to 94, 95 to 102, 104 to 108, 112, 113, 122, 123, 125, 127, 128); (h) with flagella (figure 113).

Presegmenting Parasites.—This type of parasite is so often found free, that I believe the parasite must always migrate in this stage of development.

Segmenting Parasites.—In these instances the smear must have been taken just as the corpuscle was destroyed, and before the individual segments had time to take up their separate existence.

Individual Segments.—It is not uncommon to see the parasites free in this stage of development; but it is extremely rare to find one of them with a pseudopodium (figure 96).

Ring-Form Parasites.—These parasites, on destroying the red corpuscles to which they were attached, have been freed in the form which they had assumed while surrounding a corpuscular mound (figure 15).

Ameboid Parasites.—These parasites have either been freed in this form after having destroyed the corpuscle to which they were attached, or have assumed it in preparation for a new attachment. Parasites in this phase are often difficult to distinguish from parasites with protoplasmic pseudopodia.

Compact Parasites, or Parasites Which Have Contracted Their Protoplasm into a Discoid or Spheroidal Form.—These are the types of free parasites most commonly seen both in fresh blood preparations and in stained specimens, and the compact form is, I believe, the normal resting form of the parasite when living, and that usually observed when the parasite is dead. It is in this form that the parasite is generally found in the blood of cinchonized patients, and in smears from the cadaver.

In referring to this form of the living parasite, Laveran (3) states that spherical bodies are at times free in the blood serum, and he pictures them in several stages of development. Mannaberg (4) writes: "The perfectly globular form is first assumed by the parasite when it has escaped out of the red blood corpuscle." And Marchiafava and Bignami (5), in referring to these free compact parasites, state: "We would call attention to the presence of pigmented spherules of varying sizes, which are almost always found free in the blood of a tertian patient. They are of importance in diagnosis, because sometimes they exist alone [migration?], the most careful examination failing to reveal any endoglobular parasitic bodies. These spherules are composed of a hyaline substance with small granules of freely moving pigment, and possess so characteristic an appearance to the expert eye that they quite suffice to a diagnosis of malarial tertian. For the greater part they are merely parasites which have escaped from the red corpuscle."

I have not made an exhaustive study of specimens of blood taken after the patient had been given quinine; but such smears as I have studied showed an almost complete absence of parasites. I have seen free compact parasites after the use of quinine; but I do not believe that these parasites were in migration, for they showed no such uniformity in their stages of development in any one smear as healthy parasites in migration ordinarily do, nor did they take the stain as vividly as did the healthier parasites. I believe that it was the action of the quinine that caused these parasites to abandon the red corpuscles and assume the compact form. Marchiafava and Bignami (6) state that quinine may cause certain parasites to abandon the red corpuscles, and that (7) ring-form bodies disappear from malarial blood which has been strongly cinchonized. Romanowsky (8) describes "'quinine forms' in which the clear zone of the nucleus was wanting [the corpuscular area], this structure fading insensibly into the body of the parasite"; and Golgi (9) claims that after quinine, the body of the tertian parasite becomes round and motionless. I believe that in these instances the parasites were either sick or dead.

In smears from the cadaver I have seen free parasites only in the compact form. Indeed Marchiafava and Bignami (10) go so far as to assert that "in the cadaver we do not see annular bodies; but only, as a rule, immotile discoid or spherical micrococciform bodies." In these instances I believe that the compact form is the result of the death of the parasites.

Parasites with Protoplasmic Pseudopodia.—These are parasites in varying stages of development, with attaching pseudopodia which are derived from the cytoplasm of the parasite. These pseudopodia may vary both in length and in number, and may or may not contain pigment granules. I believe that the pseudopodia are used by the parasites to secure their prey, as well as for the purpose of attachment to the red corpuscle. These attaching processes must be sharply distinguished from flagella, which arise from the chromatin substance of the parasites. Free parasites with pseudopodia are only rarely seen in stained smears from the circulating blood. They are most commonly thrown out when the parasite is free from the corpuscle; but very rarely one or more of the filaments may be seen going off beyond the periphery of the degenerated red corpuscle to which the parasite is still attached (figures 103 and 125). Dock (11) has also described and pictured the pseudopodium of a parasite going off beyond the periphery of the red corpuscle to which the parasite was attached.

Celli and Guarnieri, and later also Plehn (12), believed that they saw spores in the blood serum which "swarmed by the means of flagella" (figure 96 shows a spore with a flagellum), and Thayer (13) has observed that the "small hyaline bodies may sometimes be followed for some little distance from their original segmenting form. Under these circumstances they may show a slight dancing to-and-fro movement which suggests the possible existence of flagella."

Parasites with Flagella.—These parasites are microgametocytes in the sexual phase, the flagella arising from the chromatin substance of the parasite. In stained specimens, as a rule, the sexual flagella have a more curled appearance than do the protoplasmic attaching processes. In my experience it is rare to find pigment in connection with flagella. Flagellation may occur either when the parasite is free from the red corpuscle, or when it is still attached to it. Preflagellating parasites are occasionally found free, and may be seen with or without attaching filaments (figure 112, with attaching pseudopodia). This form of parasite is usually smaller than the segmenting bodies in the same infection, is deeply stained, and contains more chromatin in proportion to its protoplasm.

Sexual flagellating parasites are only occasionally found in stained smears from the circulating blood, but now and then a few examples may be seen. Rarely one is fortunate enough to find the complete sexual cycle.[3] I have found actively sexual parasites in all types of malaria, but only in cases showing very heavy infections. It seems to me that the term flagellata is a misnomer when applied to parasites in the sexual phase; but as it is the term which is generally accepted as applying to parasites in this phase, I continue to use it.

Sexual flagellating parasites should not be confused with parasites extruding protoplasmic pseudopodia. I believe that many of the so called flagellating bodies seen in fresh blood preparations are not parasites in the sexual phase, but parasites which, on destroying the corpuscle to which they were attached and becoming free, proceed to throw out protoplasmic processes for the purpose of capturing and reattaching themselves to other red corpuscles. It has often been noted that the red corpuscle is practically destroyed before the parasite abandons it and then proceeds to flagellate. Why should the corpuscle be destroyed before the parasite flagellates, if this flagellation is always a sexual phase? Does the parasite time its destruction of a red corpuscle to correspond with, or nearly with, the developmental stage of a sexual flagellation? I have stained some of these so called flagellating bodies and found the filaments composed of protoplasm only, and several observers have stated that the flagella observed by them appear to be protoplasmic in nature and that the nucleus does not take part in the process of flagellation. Antolisei (14) believes flagellation to be a degenerative process; the flagella he describes as sarcodic prolongations of the protoplasm. Grassi and Feletti (15) state that the nucleus does not take part in the process of flagellation, neither dividing nor entering into the flagella. Marchiafava and Bignami (16) found that "there are some flagellata in which the filaments do not contain any chromatin; but are composed of protoplasm alone. And others in which there may be one or two filaments provided with chromatin, the others being formed of protoplasm." Figure 113 shows a free flagellating parasite, with one filament composed of chromatin (a); the other filaments (o) are formed from the cytoplasm of the parasite, and pigment granules may be seen in connection with them.

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[3] Rowley-Lawson, M., *Jour. Exper. Med.*, 1911, xiii, 263.

Sakharoff (17) describes what I believe to be genuine sexual flagellating parasites. "The process of the formation of the flagellate bodies, consists in a perversion of the karyokinetic nuclear division, in a breaking up of the nucleus into the chromatin filaments, and in the escape of these from the parasite; these filaments which are in lively motion represent the flagella." Sakharoff believed this process of flagellation to be a degenerative one.

Malarial parasites in various stages of development have been noted and pictured free in the blood serum by so many observers, that it is surprising that no rational explanation has been offered to account for them. Laveran (18) constantly refers to them, stating that they may "often be found in the blood in a free state, and that at all periods of their development." Mannaberg (19) writes: "The spores which have become free . . . are often to be demonstrated in enormous numbers. . . . The amoeboid bodies too, which are developed further than their spore-forming stage, are not infrequently to be found free in the liquor sanguinis, fully retaining their structure." Thayer and Hewetson (20) state: "Occasional pigmented bodies may be found outside the red corpuscles, free in the plasma. Some of these represent full-grown bodies which have destroyed the surrounding corpuscle, while in other instances, the half-grown forms may actually leave their host." Ewing (21), in his monograph on malarial parasites, states that Celli and Guarnieri and Ziemann have depicted free parasites and that he himself has seen them in fresh blood preparations; but he believes that they are extremely rare in circulating blood. He writes: "In preparations of fresh blood, parasites so frequently pass from the cell into the plasma that it may be doubted if any accurate estimation of the number of extra-cellular bodies in the circulating blood can be obtained by this method of examination"; and Ruge (22) has pictured free parasites in several stages of development.

Bass and Johns (23) claim that the malarial parasites "cannot live for even a few minutes free in the serum." Of course this statement must apply only to parasites in artificial media or under very abnormal conditions, for all observers know that parasites are seen to remain in motion and apparently alive for many minutes free in the serum. Laveran (24) has noted that they may remain alive for half or three quarters of an hour. Marchiafava and Bignami (25) have noted that in flagellated bodies the movements of the pigment granules within the pigmented body "may continue for hours." Thayer and Hewetson (26) note that motions may be observed in flagellate bodies sometimes for as long as half an hour, the flagellating bodies being free; for they (27) state, "We have never seen the appearance of flagella in bodies still contained in the red blood corpuscles." Schaudinn (28) claimed to have watched under the microscope the "entrance into a red corpuscle" of a young parasite resulting from a segmentation, and I have frequently seen free parasites remain alive for over half an hour. The segments from a sporulating body as well as the parasites which are injected by the infecting mosquito must also be free in the blood serum for a certain length of time.

If all malarial parasites are, as I believe, attached to the external surface of the red corpuscles,[4] they must constantly be exposed to the action of the patient's serum. Stained specimens seem to show that the free parasites in migration do not remain long free, but attach themselves almost immediately to fresh red corpuscles; and if we stop to consider how short the life of the individual parasite is, even the brief periods of time when it is free in the blood serum may be comparatively long. Hence one would expect, under normal conditions for the parasite, what to us seems an immediate attachment.

The Effect of Migration, the Progressive Loss of Red Blood Corpuscles in the Intervals between Paroxysms in Tertian Malarial Infections.—Several years ago, while trying to estimate the degree of anemia in certain cases of tertian malaria showing very heavy infections, the late Dr. J. H. Donoghue of Boston and I took red counts at certain intervals, from one segmentation period to seven to eight hours after the following segmentation period, before giving quinine. The segmentation period was determined from stained specimens and not estimated from the time of the chill. We found that the anemia progressed between the paroxysms, that the greatest fall in the red corpuscle count occurred, not, as we had expected, soon after the segmentation of the parasites with the resulting destruction of red corpuscles to which they had been attached, but from six to seven hours later. Unfortunately we did not control each intermediate count of red corpuscles with a stained specimen. Later, in going over the stained specimens from these cases, I found in some of the smears certain free parasites, all in a few definite uniform stages of development. I did not at first connect these free parasites with the progress of the anemia between the paroxysms; but later I interpreted them as migratory parasites capable of destroying more than one red corpuscle. In these cases blood regeneration began as soon as quinine was given.

The maximum fall in the red count several hours after each segmentation period is due, I believe, not only to the loss of red corpuscles resulting from the segmentation of the parasites, but also to the subsequent and further destruction of corpuscles by the new group of parasites. Kelsch (29) observed the progress of anemia between the paroxysms, and stated (30) that in a robust individual, in the course of one day, the number of red corpuscles may go from normal to 1,000,000 per cubic millimeter. Dionisi (31) observed a continuous loss of red corpuscles during the afebrile interval, and Ewing (32) states that the anemia may progress during afebrile periods. I believe that quinine must be kept up for longer periods than is usual in order to insure a complete elimination of the parasites, otherwise we may be confronted with relapses. Although the malarial parasites may show a variable resistance to quinine, I do not believe that there is any stage in their development or phase in their existence when they are absolutely impervious to persistent and long continued treatment.

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[4] Rowley-Lawson, M., *Jour. Exper. Med.*, 1913, xvii, 324.

SUMMARY.

1. The malarial parasite is extracellular throughout its life cycle and migrates from red corpuscle to red corpuscle destroying each before it abandons it; in the brief intervals between, the parasite is free in the blood serum; it does not remain long free, but almost immediately attaches itself to another red corpuscle by means of delicate pseudopodia.

2. The compact form of the tertian parasite is the type of free parasite most often observed; in this form the parasite may be seen not only in migration, but after quinine and in the cadaver. I believe the compact form to be the normal resting form of the parasite, all other forms being assumed in order to secure attachment and to obtain food.

3. Care must be taken not to confound free parasites having protoplasmic pseudopodia ready for attachment with the sexual flagellating parasites, whose flagella are composed of chromatin.

4. The malarial parasite can live for some time free in the blood serum, though under normal conditions there is no reason why it should remain free for any length of time, and there are certain periods in the life of the parasite when it must be admitted that it is free from the corpuscle and survives. If the parasite is, as I believe, attached to the external surface of the red corpuscle, it is constantly exposed to the action of the patients' serum.

5. The destruction of more than one red corpuscle by each parasite would readily account for the severe and early anemia occurring in malarial infections.

6. Long continued treatment with quinine will eventually cause the death of all malarial parasites.

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EXPLANATION OF PLATES.

PLATE 61.

FREE TERTIAN MALARIAL PARASITES.

Magnification, × 1,725.

FIGS. 1 to 14. Pigmented ring-form parasites freed in the form which they had assumed when encircling corpuscular mounds. In figure 4 a very delicate pseudopodium may be seen at x. In figure 13 the periphery of the adjacent red corpuscle shows through the opening of the ring.

FIG. 15. The ring-form is seen encircling the corpuscular mound of a red corpuscle.

FIGS. 16 to 35. Various pigmented compact parasites with contracted protoplasm. Figure 20 illustrates what I believe to be a microgametocyte.

PLATE 62.

FREE TERTIAN MALARIAL PARASITES.

Magnification, × 1,725.

FIG. 36. Two segments resulting from a recent sporulating body.

FIG. 37. A young pigmented parasite with an attaching pseudopodium.

FIGS. 38 and 39. Two young pigmented parasites with contracted protoplasm.

FIG. 40. A young unpigmented parasite which has, I believe, been freed prematurely from a red corpuscle, probably by a parasite of more advanced growth destroying the corpuscle.

FIGS. 41 to 45. Young pigmented parasites with protoplasmic pseudopodia out for attachment to fresh red corpuscles. In figure 44 the parasite appears to be already attaching itself to the red corpuscle.

FIGS. 46 to 70. Pigmented parasites, ring-formed, ameboid, compact, and with protoplasmic pseudopodia.

PLATE 63.

FREE TERTIAN MALARIAL PARASITES.

Magnification, × 1,765.

FIGS. 71 to 94. Pigmented parasites with pseudopodia arising from the cytoplasm of the parasites.

PLATE 64.

FREE TERTIAN MALARIAL PARASITES.

Magnification, × 1,725.

FIGS. 95 to 108. Parasites with pseudopodia arising from the cytoplasm of the parasites. Pigment granules may be seen in connection with many of the pseudopodia.

FIG. 96. A young segment with a pseudopodium. It is rare to find segments with pseudopodia in stained specimens.

FIG. 98. The pseudopodium is seen to be perfect in form and its length is twice the diameter of a red corpuscle.

FIG. 103. The pseudopodium of the parasite is seen extending beyond the periphery of the dehemoglobinized red corpuscle to which the parasite is still attached.

PLATE 65.

FREE TERTIAN MALARIAL PARASITES.

Magnification, × 1,725.

FIG. 109. A segmented parasite free from a red corpuscle.

FIGS. 110 and 111. Presegmenting parasites free from red corpuscles.

FIG. 112. A preflagellating parasite (microgametocyte) free from the red corpuscle, with protoplasmic pseudopodia at x. Note that the parasite has been freed in the form which it had assumed when surrounding a corpuscular mound. Compare this parasite with the presegmenting parasites and note that it contains more chromatin in proportion to its cytoplasm than do the presegmenting bodies.

FIG. 113. A sexual flagellating parasite (microgametocyte) with the flagellum composed of chromatin at a, and pseudopodia from the cytoplasm at o. Granules of pigment may be seen in connection with the pseudopodia.

FIG. 114. This shows what I interpret to be a microgametocyte free from the red corpuscle. Careful examination will show what appear to be pseudopodia just starting out at the periphery of the parasite on the left.

FIGS. 115 to 120. Large free pigmented parasites. Note that the chromatin in figures 117 to 120 is very palely stained. I believe these parasites to be unpregnated adult macrogametes.

FIG. 121. An adult pigmented parasite almost freed from a red corpuscle showing advanced granular degeneration.

FIG. 122. An adult pigmented and well stained parasite which was probably about to abandon the degenerated remnant of the red corpuscle to which it was attached before the spreading of the smear. The process of smearing the blood probably separated the parasite from the remnant.

FIG. 123. An adult pigmented parasite similar to those shown in figures 117 to 120, but with a pseudopodium, suggesting that the parasite is preparing for another attachment.

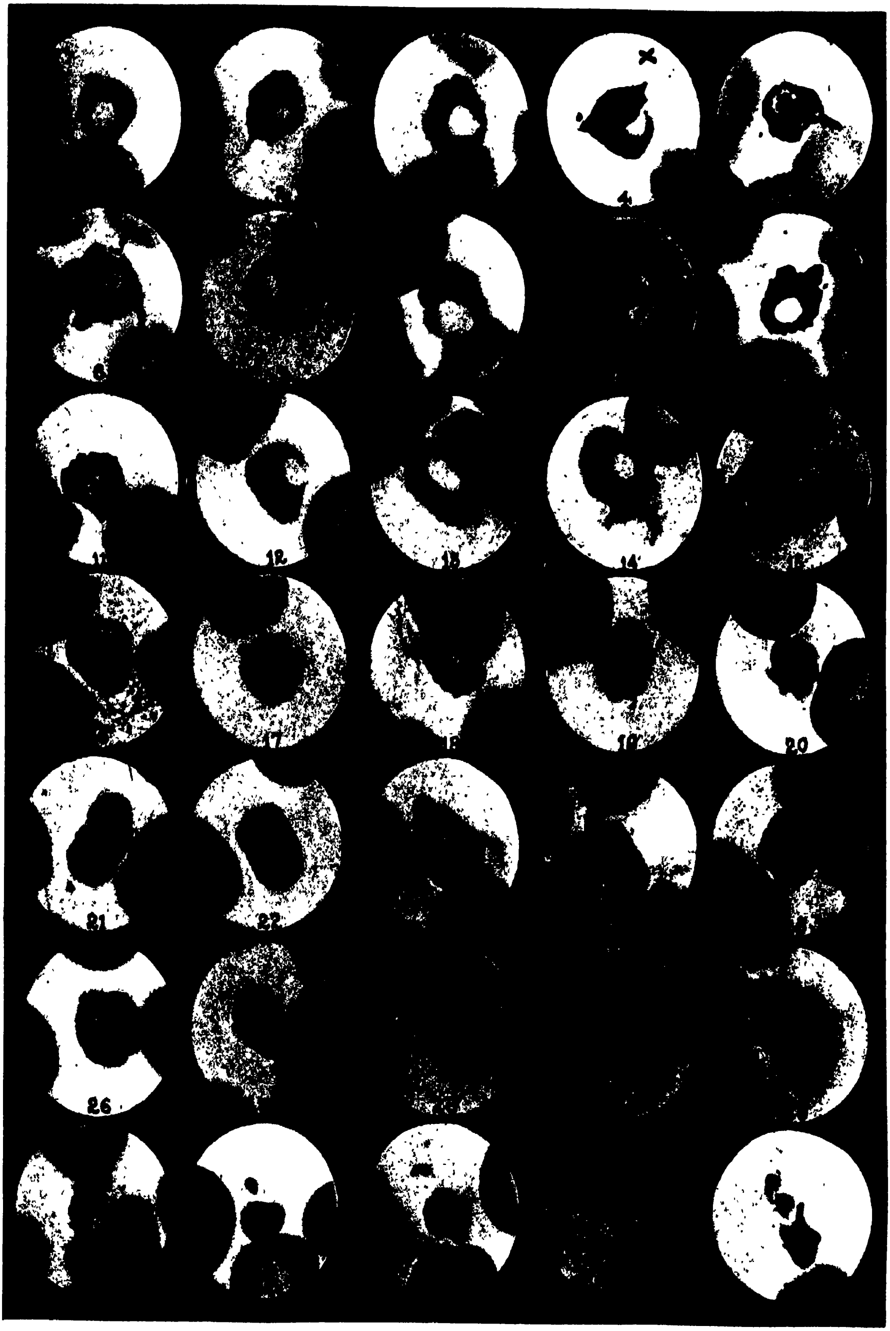
FIG. 124. Two parasites, an adult pigmented and a young unpigmented parasite, still attached to a red corpuscle showing advanced granular degeneration. I believe that the adult parasite is preparing to migrate and in doing so it will also free the young parasite, probably prematurely.

FIG. 125. A pigmented parasite preparing to migrate from a degenerated red corpuscle. Note the pseudopodium extending beyond the periphery of the red corpuscle.

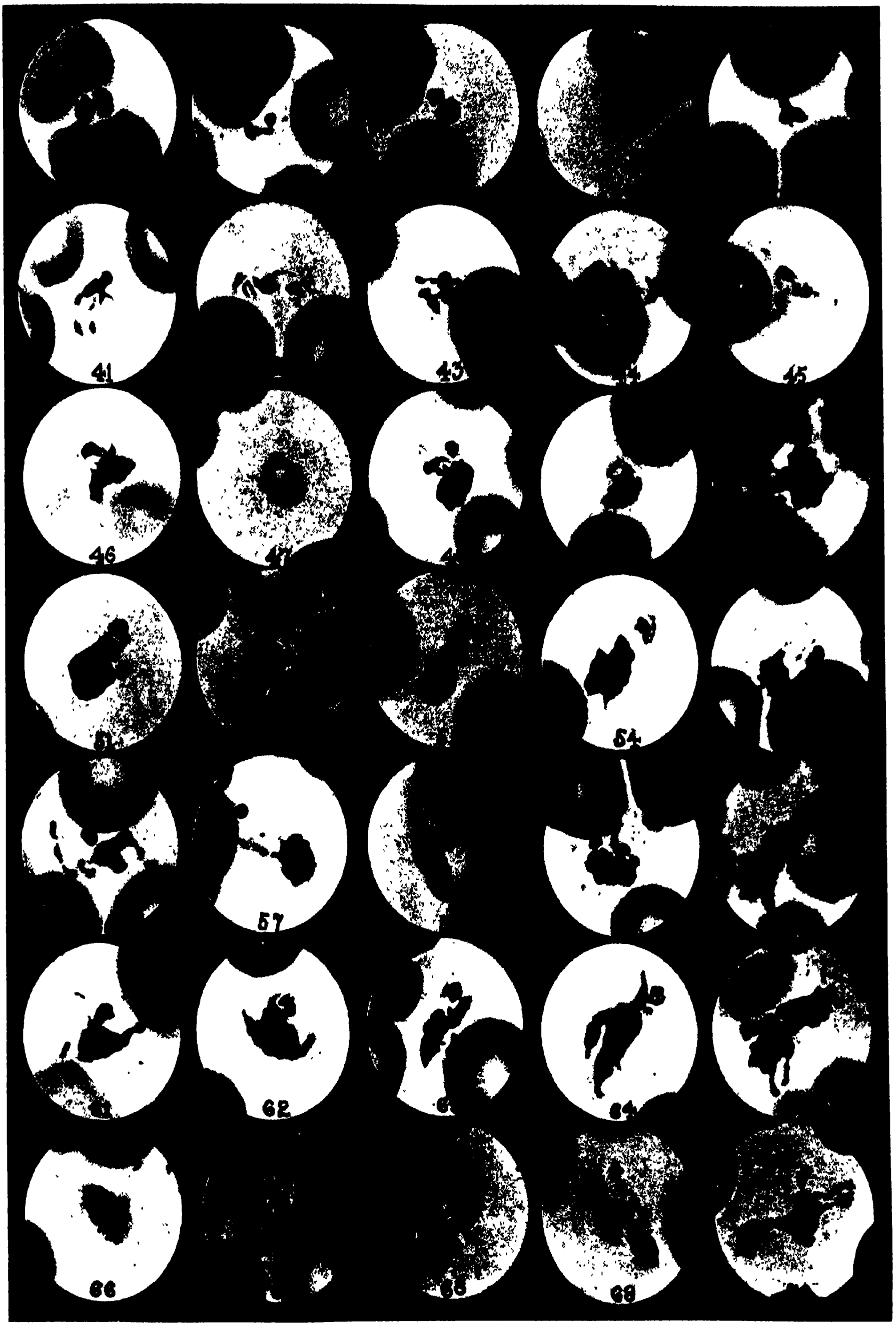
FIG. 126. A young parasite which appears to be only just freed from the remnant of a degenerated red corpuscle, shown just below the parasite.

FIG. 127. A free parasite with protoplasmic pseudopodia.

FIG. 128. A free pigmented parasite with protoplasmic pseudopodia.

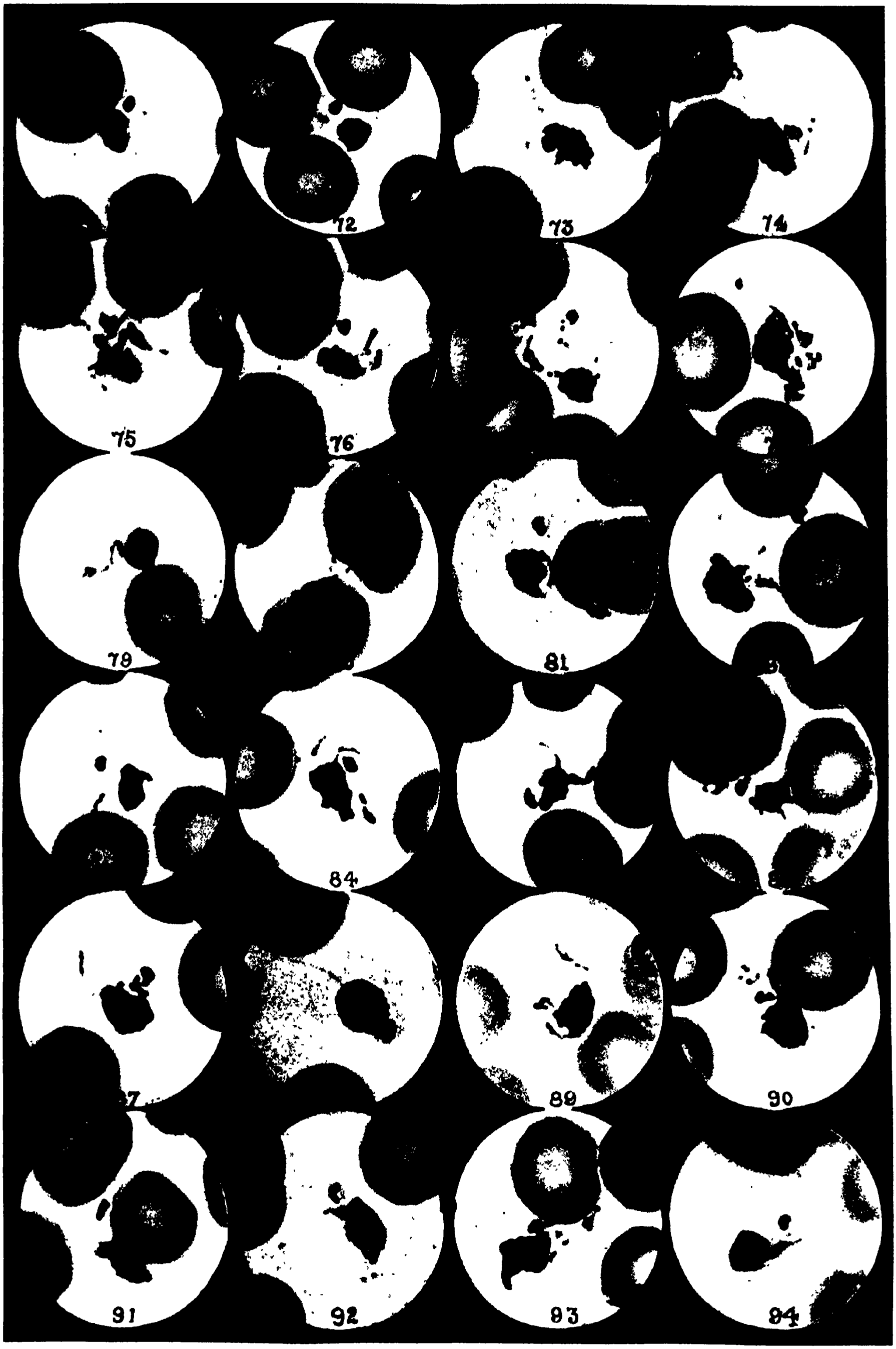


(Lawson: Free Malarial Parasites.)

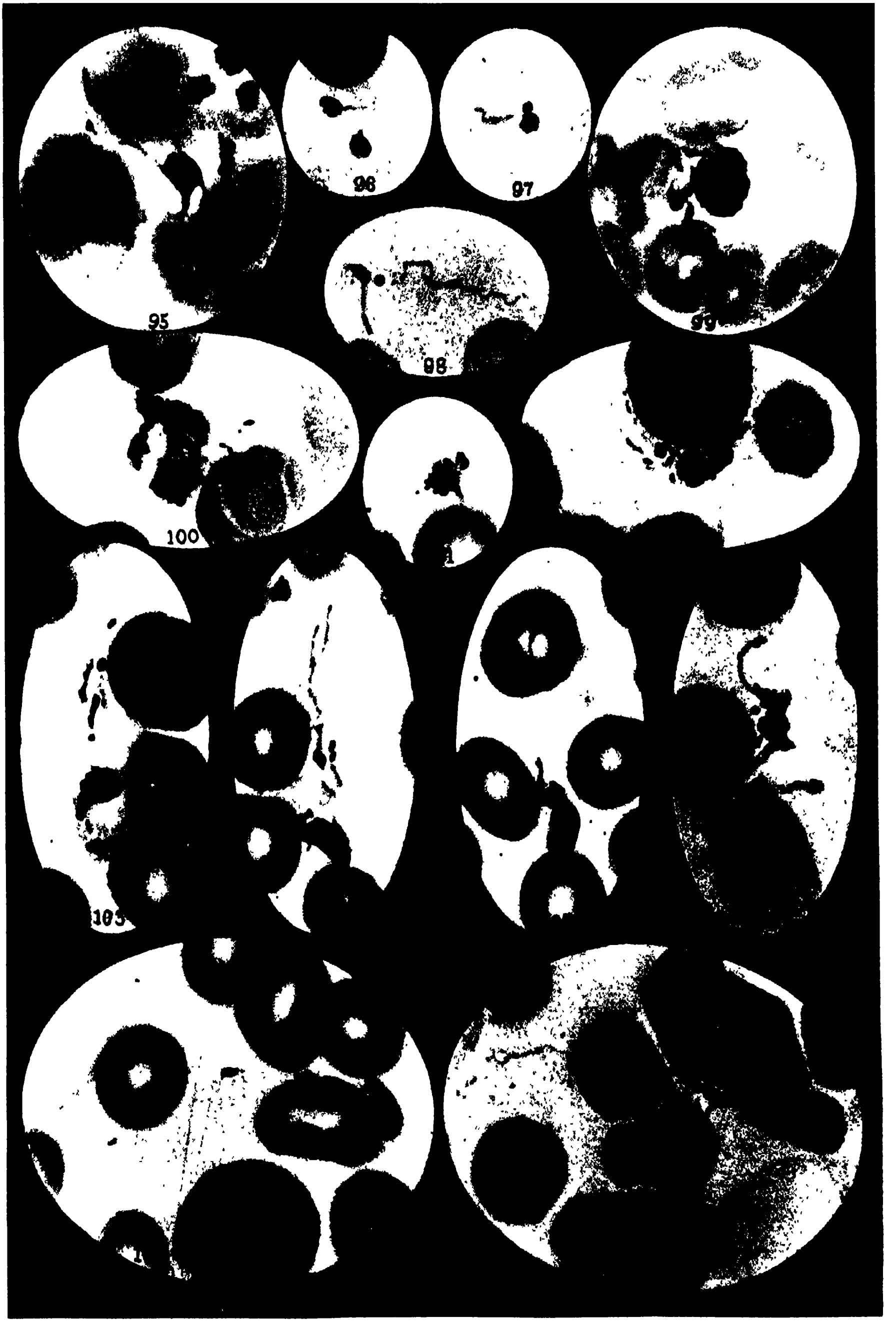


(Lawson: Free Malarial Parasites.)

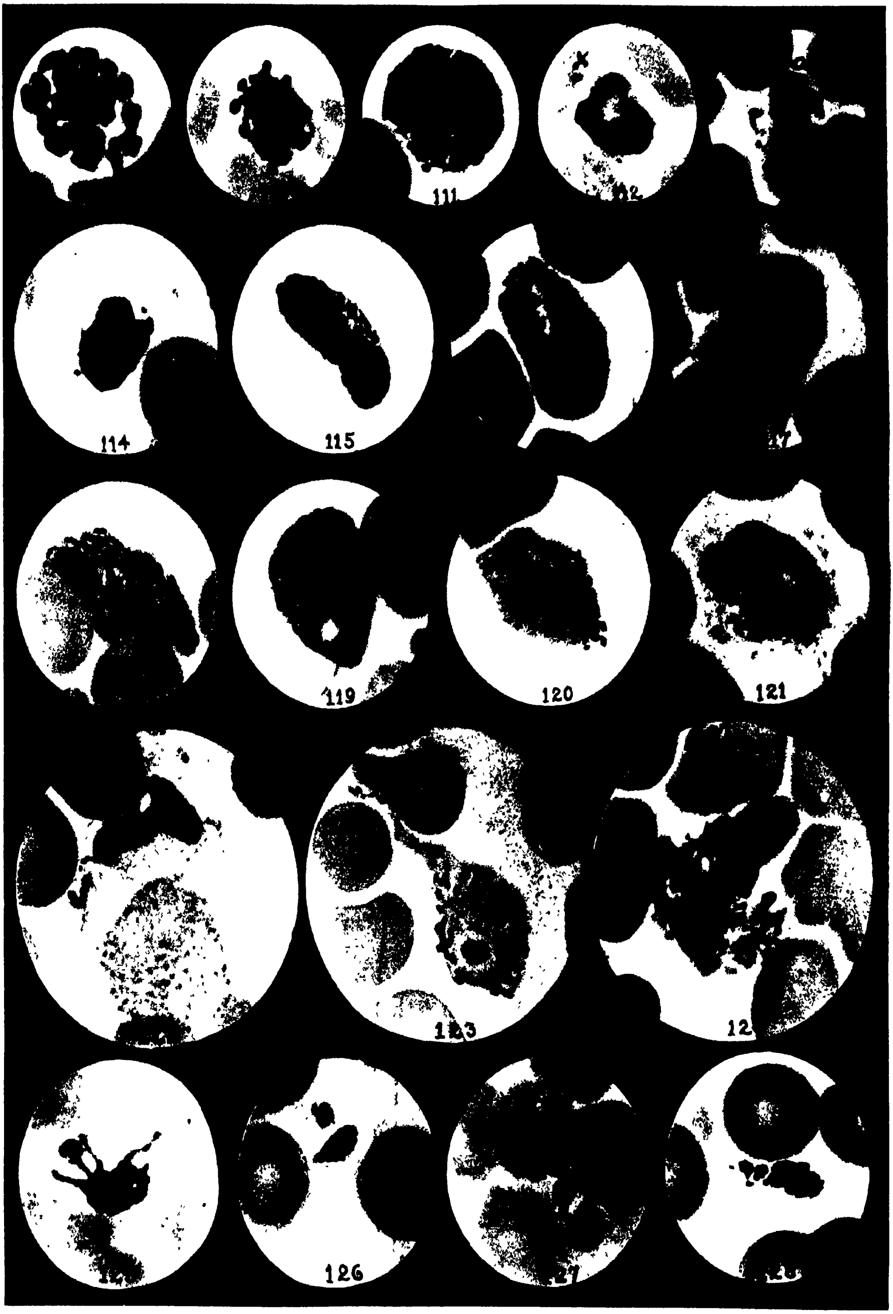
PLATE 63.



(Lawson: Free Malarial Parasites.)



(Lawson: Free Malarial Parasites.)



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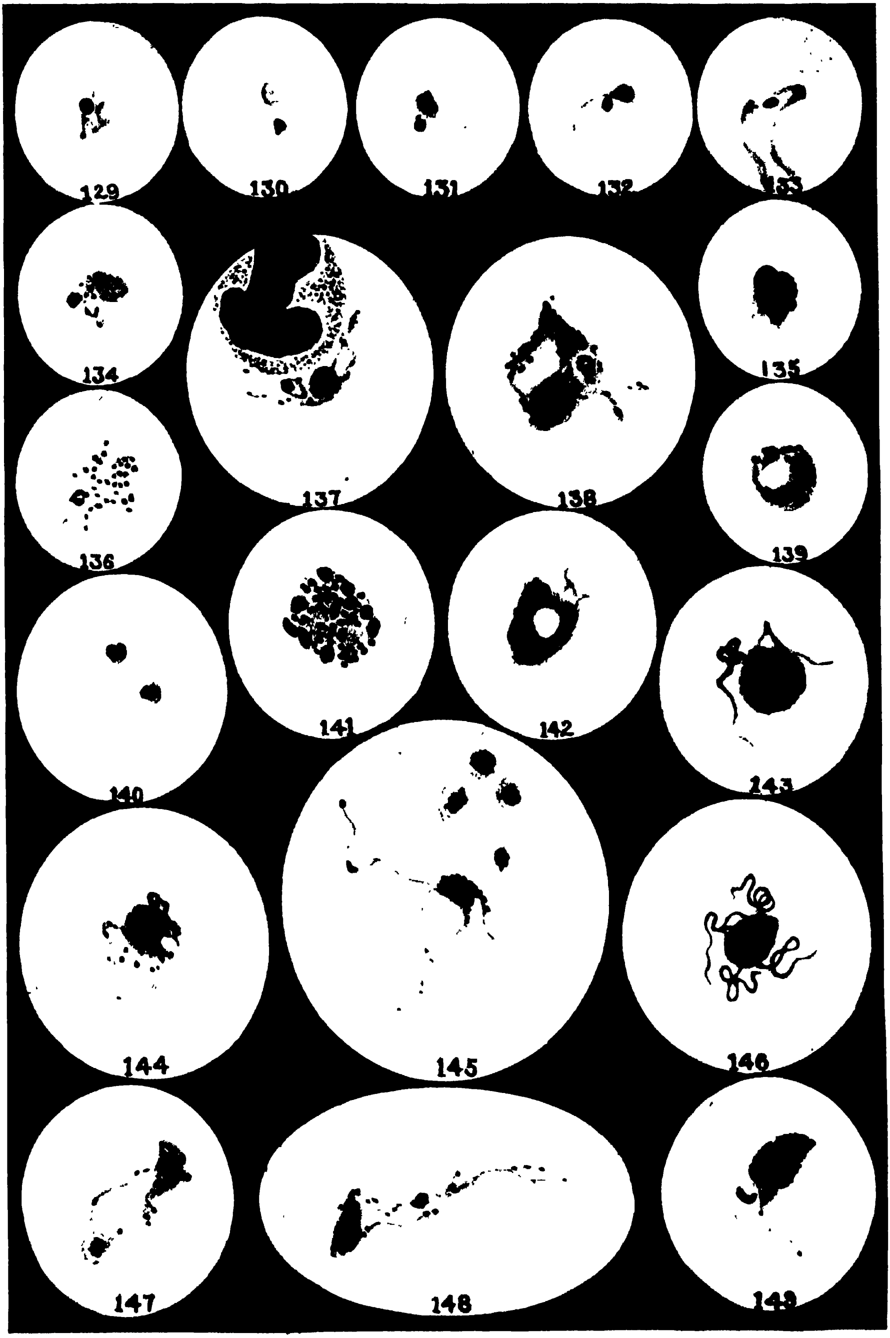

(Lawson: Free Malarial Parasites.)

PLATE 66.

FREE TERTIAN MALARIAL PARASITES.

FIGS. 129 to 133. (Correspond to figures 43, 42, 45, 37, and 44.) Young pigmented parasites with protoplasmic pseudopodia. In figure 133 the parasite appears to be attaching itself to the red corpuscle.

FIG. 134. (Corresponds to figure 48.) A pigmented parasite, freed with the pseudopodia in the form of a loop, which had been used to encircle a corpuscular mound.

FIG. 135. (Corresponds to figure 20.) A compact pigmented parasite.

FIG. 136. (Corresponds to figure 75.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 137. (Corresponds to figure 102.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 138. (Corresponds to figure 100.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 139. (Corresponds to figure 7.) A pigmented ring-form parasite, freed in the form which it had assumed when encircling a corpuscular mound.

FIG. 140. (Corresponds to figure 96.) A segment resulting from a recent sporulation, with a pseudopodium. It is rare to find one of these young parasites with a pseudopodium.

FIG. 141. A free presegmenting parasite.

FIG. 142. (Corresponds to figure 112.) A preflagellating parasite (microgametocyte) with pseudopodia. The parasite has been freed in the ring-form which it had assumed when encircling a corpuscular mound. Compare this parasite with the presegmenting body.

FIG. 143. (Corresponds to figure 99.) A pigmented parasite with pseudopodia arising from the cytoplasm of the parasite.

FIG. 144. (Corresponds to figure 113.) A sexual flagellating parasite (microgametocyte) with flagellum composed of chromatin substance, and pseudopodia (attaching processes) composed of the cytoplasm of the parasite.

FIG. 145. (Corresponds to figure 95.) A pigmented parasite with pseudopodia (attaching processes) arising from the cytoplasm of the parasite. Pigment granules can be seen in connection with some of the filaments. The other bodies seen in the picture are blood plates.

FIG. 146. A sexual flagellating parasite (microgametocyte), the flagella being derived from the chromatin substance of the parasite. Compare this parasite with the parasites with attaching pseudopodia. The attaching pseudopodia arise always from the cytoplasm of the parasite.

FIG. 147. A pigmented parasite with the pseudopodia in the form of a loop, which formerly was used to surround a corpuscular mound.

FIG. 148. (Corresponds to figure 104.) A pigmented parasite with attaching pseudopodia arising from the cytoplasm of the parasite.

FIG. 149. (Corresponds to figure 92.) Pigmented parasite with attaching pseudopodia arising from the cytoplasm of the parasite.

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MORPHOLOGICAL AND DEVELOPMENTAL ANOMALIES OF A PATHOGENIC STRAIN OF TRYPANOSOMA LEWISI AND THEIR RELATION TO ITS VIRULENCE.*

BY WADE H. BROWN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 67 AND 68.

The occasional references to unusual manifestations of virulence and the numerous descriptions of morphological and developmental anomalies of *Trypanosoma lewisi* contain scarcely a suggestion of a connection existing between changes in the virulence and changes in the morphology and developmental tendencies of the organism. The first suggestion of the possibility of such a relation is contained in the work of Wendelstadt and Fellmer,[1] on the effect of the passage of *Trypanosoma lewisi* through cold-blooded animals. These authors noted an increase in the virulence of the trypanosome for rats, and, at the same time, a profound alteration in the morphology of the organism, the main feature of which was a marked prolongation of the posterior extremity.

In a recent article[2] I published an account of an unusual increase in the virulence of a pathogenic strain of *Trypanosoma lewisi*. As this strain showed many morphological and developmental peculiarities, the life cycle and morphology of the organism were studied in eighty-two rats, with especial reference to the nature of the infection. In different series of infections, the rate of passage and the dose of trypanosomes were varied in an attempt to modify the resulting infections. As a control to this series, a study was made of a parallel series of rats infected with a typical benign strain of

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* Received for publication, April 13, 1914.

[1] Wendelstadt, H., and Fellmer, T., *Ztschr. f. Immunitätsforsch., Orig.*, 1909, iii, 422; 1910, v, 337.

[2] Brown, W. H., *Jour. Exper. Med.*, 1914, xix, 406.

Trypanosoma lewisi that showed no morphological or developmental peculiarities. The details of these experiments and the effect of such procedures, as indicated, upon the course of the infection will be presented in another paper.

The usual life cycle and morphology of *Trypanosoma lewisi* in the rat's blood are so well known that they require no description, and it is not my purpose to present an exhaustive study of the anomalies of this organism and the factors giving rise to them, but rather the relation of anomalous development and morphology to the virulence exhibited by this particular strain. Therefore, only those deviations from the normal will be described that were pronounced and occurred with sufficient frequence in both fresh and stained preparations to be regarded as characteristic of the strain or of a particular infection. Most, if not all, of the peculiarities of this strain have been recognized by other workers but some of them are comparatively rare and apparently have not all been recognized in a single strain, while the conditions giving rise to these anomalies are but little understood.

DEVELOPMENTAL ANOMALIES.

In addition to the usual forms of multiplication, two clearly differentiated types of longitudinal division were observed; both of these were examples of equal binary fission; in rare instances the division was unequal or was multiple. The first of these types of division (figures 1 and 2) is more closely related to the usual process of multiplication as it occurred in trypanosomes showing the increased size, granular and basophilic cytoplasm characteristic of this period in the life cycle of the organism. Such forms of division were never numerous and were observed only during the usual period of multiplication. The sequence in the process of division could not be determined with certainty, as our only means of recognizing these forms comes with the division of the flagellum and undulating membrane. It is certain that division of these structures may occur first, but it is impossible to say whether trypanosomes with a divided nucleus or blepharoplast may not ultimately divide in this manner.

The second type of longitudinal division (figures 3 to 7) occurred far more frequently than the first and was observed during all phases of the blood infection. These forms presented a great variety of sizes, occurring in organisms that were quite large as well as in exceedingly small ones, but the general contour, nature of the cytoplasm, and staining reactions of all were characteristic of adult trypanosomes. A study of a large series of these forms indicated that the usual sequence of division was flagellum, undulating membrane, nucleus, and blepharoplast; but again, the sequence was undoubtedly subject to variation.

Several examples of multiple longitudinal division of adult trypanosomes were seen, one of which is shown in figure 7, but unequal division of this type of organism was not observed.

From published accounts of longitudinal division of *Trypanosoma lewisi,* it is uncertain whether both of these types of division have been recognized; the second is undoubtedly the one that is generally described. In a sense, the two types might be regarded as but one, since they grade into each other and probably represent a common tendency initiated in the young trypanosome and persisting in the adult organism even after all other forms of multiplication have disappeared from the blood.

The almost endless variety of forms exhibited by *Trypanosoma lewisi* in the usual process of multiplication precludes any consideration of the morphology of such organisms except as regards the degree of irregularity. Further, during the period of multiplication variations in the rate, extent, and persistence of multiplication may occur which markedly alter the character of the infection. At least two types of deviation from the normal have been recognized as indicating an increased severity of infection; either multiplication may be very rapid and marked with great numbers of irregular forms, especially small and imperfectly developed trypanosomes, or, with a normal rate and type of multiplication, this period of the life cycle does not cease at the usual time but continues on through the second or even the third week of the infection. According to our experience, the first type of infection is typically acute and occasionally terminates fatally at the height of multiplication, while the second, although usually more severe, progresses more slowly, and fatal terminations occur, as a rule, after the second week of the infection.

ATYPICAL MORPHOLOGY.

Adult forms of *Trypanosoma lewisi* are generally uniform in character. The variations that we noted concerned the size, the nature of the flagellum and undulating membrane, the character of the posterior extremity, and the nucleus and the blepharoplast of the trypanosome.

Extremely small trypanosomes, measuring no more than 7 to 8 micra from the tip of the posterior extremity to the origin of the free flagellum, but possessing all the other characteristics of the normal adult trypanosome, were numerous in some severe infections. These small trypanosomes (figures 8 and 9) are of especial interest, as they appear to be identical with the small forms regarded by Swellengrebel and Strickland[3] as peculiar to the life cycle of *Trypanosoma lewisi* in the invertebrate host.

The posterior elongated forms of *Trypanosoma lewisi* described by many authors, and erroneously regarded by some as "of constant occurrence and very numerous at a certain stage of the multiplication-period,"[4] were very numerous in this strain of the organism. In some instances 10 per cent. of the trypanosomes showed this peculiarity (figure 10). Two types of elongated extremity were observed with about equal frequency. One of these possessed a delicately pointed tip (figures 11 and 12), and the other a bulbous extremity (figures 3 and 13). These posterior elongated forms were observed during all periods of the infection, but were most numerous during the latter part and immediately following the multiplication period. While this variety of trypanosome was usually larger than the normal adult, extremely small and irregular forms, such as those in figures 14 and 15, were occasionally seen. Other peculiarities of structure that usually accompanied an elongation of the posterior extremity were a highly developed and plicated undulating membrane and a short free flagellum, both of which are shown typically in figure 11. In a few fortunately stained preparations of these organisms a series of extremely faint longitudinal striations were seen that suggested the presence of myonemes.

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[3] Strickland, C., and Swellengrebel, N. H., *Parasitol.*, 1910, iii, 436.

[4] Minchin, E. A., An Introduction to the Study of the Protozoa, London, 1912, 292.

Trypanosomes with an abnormally short posterior extremity (figure 16) were not infrequently observed along with the elongated variety. Occasionally there was a virtual absence of the post-blepharoplastic segment, the blepharoplast being practically at the tip of the extremity.

The undulating membrane and the flagellum of *Trypanosoma lewisi* may vary quite independently of the posterior extremity. The two trypanosomes in figure 17 show extremes in the development of the undulating membrane, while figure 18 shows an organism in an early stage of division with an unusually prominent membrane. Figure 19 illustrates an extreme shortening of the free flagellum in an otherwise peculiar trypanosome.

Innumerable irregularities of the nucleus and blepharoplast have been described in detail by various authors and most of them are properly regarded as involution or degeneration phenomena. I observed total absence of a stainable nucleus, resulting from atypical division, in a few instances. This anomaly occurred in young trypanosomes with no evidence of degeneration, as well as in adult organisms where degeneration could not be excluded.

The production of ablepharoplastic trypanosomes through the agency of drugs has attracted so much attention that the spontaneous occurrence of this anomaly in *Trypanosoma lewisi* deserves especial mention. Figures 20 to 22 show ablepharoplastic trypanosomes that are either young or early multiplication forms with no evidence of degeneration or mechanical distortion. Absence of a blepharoplast was noted in all types of *Trypanosoma lewisi,* but was most frequent in the adult organism. While in most instances there was not the slightest suggestion of a blepharoplast, occasionally there was an extremely small granule (figure 20), representing a rudimentary blepharoplast. Although this type of organism was constant, and even numerous in many infections, it could not be found in other infections of the same strain of *Trypanosoma lewisi.* Examples of the developmental and morphological anomalies of this strain might be greatly extended, but these are sufficient to show the unusual character of the strain.

RELATION OF DEVELOPMENTAL AND MORPHOLOGICAL ANOMALIES TO VIRULENCE.

In studying these features of the organism with reference to the character of the infection, as indicated earlier in this paper, certain facts were brought out that indicated a degree of correspondence between the occurrence of developmental and morphological anomalies and the virulence of the strains with which I was working. As the blood of each of the eighty-two rats infected with our pathogenic strain of *Trypanosoma lewisi* showed most of the anomalies of development and morphology that have been described, these features may be regarded as characteristic of the strain. The control strain which, when first isolated, produced very benign infections and showed no anomalies, subsequently showed an increased virulence in certain series of infections coincident with the appearance of anomalous forms of trypanosomes in the blood of infected rats. The simultaneous alteration of these several properties of the organism suggests something more definite than a mere coincidence.

Further, in both strains the relative numbers and the variety of atypical trypanosomes differed in different infections and were greatest in two classes of infections, both of which have already been described as atypical infections. In one of these the incubation period was short, multiplication was rapid and pronounced, and there were enormous numbers of trypanosomes in the peripheral circulation. Some of these infections were unusually severe and occasionally terminated fatally, while others, in which multiplication ceased early and abruptly, showed a very mild course throughout.

The second class of infections in which anomalies were prominent differed from the first in that the evolution of the infection was more gradual and the period of multiplication persistent. The prominent features of these infections were the persistence of multiplication and the severity of the infection.

In conclusion, then, while it was certain that an intimate relation existed between the developmental tendencies and the morphology of these strains of *Trypanosoma lewisi,* it could not be definitely determined to what degree these characteristics were coördinated with the virulence. There were some facts that might lead one to believe that as virulence influenced the course of the infections it also exercised an influence upon the developmental tendencies and hence the morphology of the organisms. My observations, however, inclined me to the opinion that, in so far as *Trypanosoma lewisi* was concerned, developmental tendencies exercised the dominant influence upon the morphology of the organism, and, while these characteristics did not correspond rigidly with the virulence of the organism, that anomalies of development and morphology, to some degree, corresponded with the virulence as manifested in the infections of *Trypanosoma lewisi* that I studied.

SUMMARY.

1. The morphological and developmental anomalies of a pathogenic strain of *Trypanosoma lewisi* have been described to show the unusual character of the strain.
2. Especial attention is called to the spontaneous occurrence of ablepharoplastic forms of *Trypanosoma lewisi.*
3. It is pointed out that morphological anomalies were most pronounced in infections that showed unusual conditions of multiplication, and that such infections usually proved severe.
4. Finally, an appreciable correlation between the morphological and developmental characteristics and the virulence as manifested in these examples of infection with *Trypanosoma lewisi* has been suggested.

EXPLANATION OF PLATES.

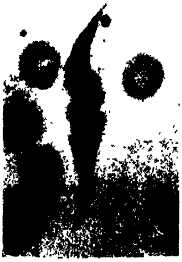
Plate 67.

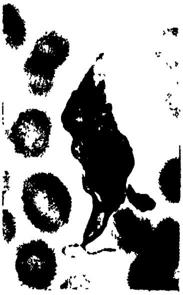
The photomicrographs show a magnification of 1,000 diameters, except figure 10, in which the magnification is 1,050. Figures 1, 3, 5, 7, 10, 16, 18, 21, and 22 are from smears of rat blood stained with Wright's blood stain. All the other figures are from similar preparations stained with Giemsa stain. The figures are not retouched.

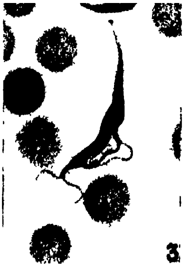
Figs. 1 to 7. Longitudinal division of *Trypanosoma lewisi.* Figures 1 and 2 show trypanosomes with the usual cytoplasmic characteristics of organisms in process of multiplication, while figures 3 to 7 represent division of older forms. Figure 3 shows division of the flagellum in a trypanosome with an elongated posterior extremity, and figure 7 multiple division of an adult trypanosome.

Figs. 8 and 9. Small trypanosomes with the characteristics of the adult organism.

Figs. 10 to 15. Trypanosomes with an elongated posterior extremity.







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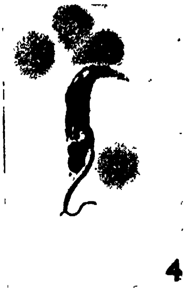

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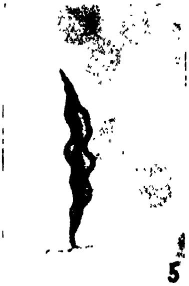

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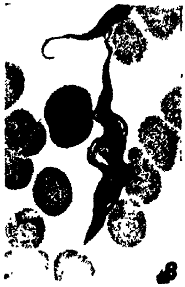

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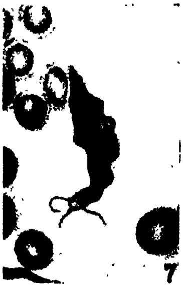

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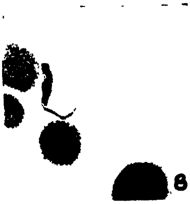

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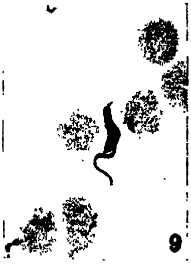

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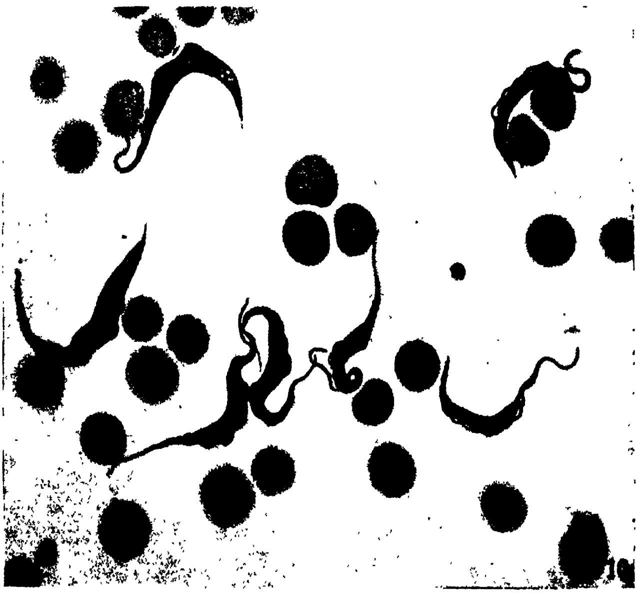

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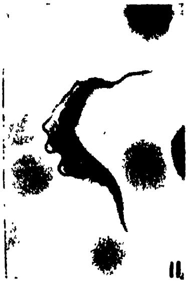
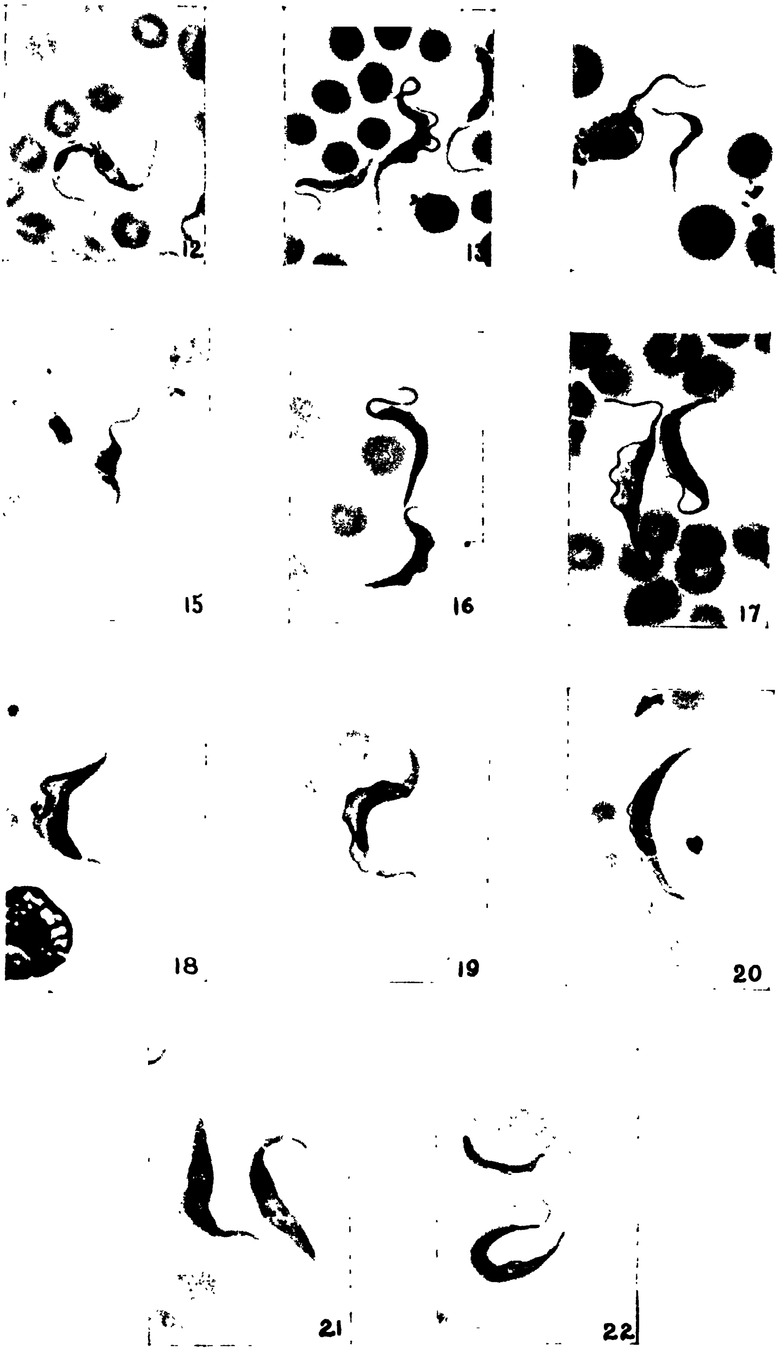


11

(Brown: Anomalies of Pathogenic Strain of *Trypanosoma lewisi.*)



(Brown: Anomalies of Pathogenic Strain of *Trypanosoma lewisi*.)

FIG. 10. Three adult trypanosomes with posterior elongations. Compare these with the two young forms, also with elongated posterior extremities, and with the normal adult trypanosomes.

FIG. 11. A very large trypanosome with a delicately pointed posterior extremity and a highly developed undulating membrane.

PLATE 68.

FIG. 12. An extreme elongation of the posterior extremity.

FIG. 13. Posterior extremity with a bulbous tip. Compare with figure 3.

FIG. 14. Small anuclear trypanosome.

FIG. 15. A young trypanosome with posterior elongation.

FIG. 16. Short posterior extremity in an adult trypanosome.

FIGS. 17 to 19. Trypanosomes with highly developed undulating membrane. Compare with figures 11 and 13.

FIG. 20. *Trypanosoma lewisi* with a rudimentary blepharoplast. Only the dot at the root of the flagellum represents the blepharoplast. The dark line is the result of heavy staining of the flagellum crossing the body of the organism. Compare with figure 11.

FIG. 21. An ablepharoplastic trypanosome.

FIG. 22. An ablepharoplastic trypanosome with an elongated posterior extremity.

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ON CERTAIN SPONTANEOUS CHICKEN TUMORS AS MANIFESTATIONS OF A SINGLE DISEASE.

I. SPINDLE-CELLED SARCOMATA RIFTED WITH BLOOD SINUSES.*

BY PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 69 TO 71.

Recently three transplantable chicken tumors distinct in character have been found to have a filterable cause.[1] The differences between these tumors are traceable to differences in the causative agents. Each agent gives rise in normal fowls to tumors of the sort from which it was isolated by filtration, and to tumors of this sort only. For example, the agent derived from a transplantable osteochondrosarcoma gives rise to sarcomatous tumors in which cartilage and bone are laid down. Certain minor variations, it is true, do occur in each tumor strain as intercurrent phenomena. The cells of the sarcoma known in our laboratory as Chicken Tumor I are, in some chickens, of very attenuated spindle form, again oat-shaped or almost round, again interspersed with sarcomatous giant cells; and the course of the disease varies somewhat in individual fowls. But the growth is always a spindle-celled sarcoma, and its modifications are not greater than those observed in certain rat and mouse tumors propagated only by transplantation and dependent on the survival of a single race of cells. Attempts to bring about variations by injuring the filterable agent have been unsuccessful, as have attempts to make it affect epithelium.

There is good ground to suppose that other tumors of the fowl besides those already studied are caused by filterable agents. The range in structure and behavior among chicken tumors is very wide.

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* Received for publication, April 20, 1914.

[1] Rous, P., *Jour. Am. Med. Assn.*, 1911, lvi, 198. Rous, P., and Murphy, Jas. B., *Jour. Exper. Med.*, 1914, xix, 52.

Even when composed of cells of similar origin they often exhibit, like mammalian growths, a strikingly various structure and course. Must one suppose a distinct causative agent not only for each type of neoplasm as determined morphologically, but for the almost infinite number of variations in structure and behavior of such types? The present article and the one following it deal with this point. Briefly, it has been found that two spontaneous chicken tumors recently transplanted have each given rise to neoplasms identical in composite behavior with a tumor strain already under propagation. As will be shown in the present paper, the spontaneous tumor known as Chicken Tumor 38 of our series, seems to be a manifestation of a disease-complex already reported upon and known as Chicken Tumor 18.[2] This latter growth is a spindle-celled sarcoma, rifted in a characteristic manner with blood sinuses and tending to metastasize to the muscles, especially in the neighborhood of joints.

THE SPONTANEOUS TUMORS.

The spontaneous tumor No. 38 resembled the spontaneous tumor No. 18 only in the fact that it was a growth composed of spindle cells of connective tissue origin. The fowl carrying it was a well grown but emaciated Plymouth Rock hen. It was brought to the laboratory while yet alive. The irregular tumor mass, situated in the subcutaneous tissue between the left leg and the body, measured 10 by 6 by 5 centimeters, was imperfectly encapsulated, attached to the sheath of the thigh muscles, and just beginning to involve the skin. Strands extended between the leg muscles into the drumstick. At the center of the mass was a cavity with ragged walls, containing about forty cubic centimeters of clear, straw-colored fluid. The tumor tissue was finely striated, pinkish white, rather soft, and varied with many irregular, translucent areas of colliquation. There were no metastases. Histologically the growth was composed of strands of attenuated spindle cells with much collagen, sometimes in the form of ribbons (figure 1). A few round cells were scattered here and there in the growth. There was very little resemblance to the spontaneous tumor No. 18. This latter occurred in the gizzard of a brown Leghorn fowl and metastasized to several points in the skeletal muscles. Both the primary and secondary tumors consisted of a very regular spindle-celled tissue, rifted to an extraordinary degree with blood sinuses into which the growth showed a tendency to extend, with result in an intracanalicular arrangement.

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[2] Rous, P., and Lange, L. B., *Jour. Exper. Med.*, 1913, xviii, 651.

The fowl carrying Chicken Tumor 38 was killed and bits of the neoplastic tissue were implanted in the breast muscle of two normal Plymouth Rock fowls, in both of which a growth slowly developed. With repeated passage the tumor's rate of growth has increased somewhat, but like No. 18 it is still much less malignant than the simple spindle-celled sarcomata, Nos. 1 and 43. It is now growing in its fourth successive series of hosts. A filterable agent causing it, distinct from the tissue cells, has been demonstrated by three methods; namely, by drying, by glycerination, and by filtration through Berkefeld cylinders impermeable to small bacteria. The findings compared with those in the case of No. 18 are, briefly, as follows:—

COMPARISON OF THE TUMOR STRAINS.

Both growths, whether obtained by transplantation or by the action of the filterable agent as such, are, in the gross, solid, pinkish white, unencapsulated, firm, and markedly resistant to the knife. When growing in voluntary muscle they tend to bind the fibers and limit motion, a feature not observed in the case of the simple spindle-celled sarcomata already mentioned. Both growths are composed of attenuated spindle cells arranged, often very regularly, in bundles or strands with much collagen which is usually in the form of bands or ribbons (figure 2). Giant cells are not present. The rifting with blood sinuses, which was so important a feature of the earlier generations of Chicken Tumor 18, is now only occasionally seen in this growth. Absent at first from Chicken Tumor 38, it has recently been met with in several cases (figure 3). Histologically the two growths are at present practically indistinguishable.

Chicken Tumor 18 in its earlier generations showed a notable tendency to metastasize to the skeletal muscles, especially in the neighborhood of joints. The lungs, heart, and liver were affected sometimes, though rarely. Among nine fowls which have thus far died of Tumor 38, one had secondary tumors in lung, liver, and gizzard. In three cases there were metastases in the skeletal muscles. The simple, spindle-celled sarcomata, Nos. 1 and 43, have never shown this feature. Of the three instances referred to, one had a nodule in the wing, a second both wing and leg metastases, while in the third the involvement was widespread. A small nodule was present in the lung, larger secondary growths in the muscles of the neck, legs, and hip, and a series of coalescing masses connected the junctions of the sternal and vertebral ribs, forming what may be called a neoplastic rosary (figure 5). The whole condition closely resembled that in a fowl which died of tumors produced by the injection of a filtrate of Chicken Tumor 18.[3]

Tumor 18, though spontaneous in a brown Leghorn fowl, grows much better in the Plymouth Rock variety, a fact only recently determined and still to be reported upon in full. Chicken Tumor 38 likewise succeeds better in Plymouth Rocks than in brown Leghorns. But the two cases are hardly to be compared, for Tumor 38 occurred spontaneously in a Plymouth Rock fowl, and, from what is known of the laws governing transplantation, might be expected to succeed best in hosts of this sort, as indeed it does. Tumor 18 acts against the rule, growing better in hosts of an alien variety.

THE FILTERABLE AGENTS.

A causative agent for Chicken Tumor 18, as distinct from the cells, has been demonstrated only by filtration experiments. The dried or glycerinated tumor tissue is incapable of causing the growth. Tissue of No. 38, dried or glycerinated, gives rise to the tumor in a considerable percentage of normal fowls injected, and this within a few weeks. The Berkefeld filtrate of an extract of the growth acts almost as quickly. Filtrates derived from No. 18, on the other hand, seldom cause a tumor until several months after the injection. The differences in resistance and activity of the causative agents, as thus indicated, are the only points of dissimilarity between the tumors at present.

[3] Rous and Lange, *loc. cit.*

CHICKEN TUMOR 27.

All in all, the findings give one good reason to suppose that the spontaneous chicken tumors, Nos. 18 and 38, are different manifestations of a single disease-complex. It has seemed possible that other expressions of this complex might be present among our forty-five spontaneous neoplasms of the fowl. A search shows that the growth known as No. 27 is probably such a case. The host, a brown Leghorn hen, had several large lumps in the muscles of the wings and legs which limited motion markedly, a small nodule in the gizzard, and a number of raised, sharply defined, plateau-like masses in the skin, some deeply pitted with feather follicles and one of them ulcerated. It was impossible to say which growth was primary. All consisted of a close textured, finely striated, firm, pink, sarcomatous tissue. At the time, the case appeared unique, and indeed among the spontaneous growths subsequently obtained none has given a similar picture. But among the many fowls dying of transplantation tumors of No. 18,—now in its eleventh successive series of hosts,—a single instance closely resembling that of No. 27 has been met with. The fowl, of the second transplantation generation, is mentioned in a previous article.[4] The discoid masses in the skin consisted, as in the case of Tumor 27, of a sharply defined, nearly homogeneous, spindle-celled, sarcomatous tissue in the looser layers of the corium, the masses in the muscle of the same sarcoma arranged for the most part in the familiar intracanalicular pattern (figure 4). Unfortunately no adequate attempt was made to transplant Tumor 27. The other spontaneous chicken tumors do not suggest, even remotely, the disease-complex of Nos. 18 and 38.

SUMMARY.

Two spontaneous chicken tumors, unlike in several important respects, have given rise on transplantation to neoplasms of identical character. The spontaneous growth, No. 18, situated in the gizzard, was a spindle-celled sarcoma rifted with blood sinuses into which it extended, with result in what may be described as an intracanalicular pattern. The metastases, which were in the voluntary muscles, showed the same peculiar structure. Tumor 38, occurring in the subcutaneous tissue of the groin, was a solid, spindle-celled sarcoma of rather close texture, with few blood vessels. Here and there were small areas of softening, and at its center was a large degeneration cyst with ragged walls, containing a clear fluid. There were no metastases. The transplantation tumors from both growths have been characterized by slow growth, tendency to metastasize to the skeletal muscles without involvement of the lungs, and a structure which at one time is that of a very regular spindle-celled sarcoma containing many bands and ribbons of collagen, and at another that of a sarcoma rifted with blood sinuses like the spontaneous tumor No. 18. At present the two strains are practically indistinguishable in appearance and general behavior. Both are caused by filterable agents. The agent causing No. 38, unlike that causing No. 18, retains its activity in tumor tissue which has been dried or glycerinated; and in a Berkefeld filtrate it is much the more active in causing tumors. These differences can hardly be thought of as constituting a fundamental distinction between agents which, to judge from their effects, are almost undoubtedly different strains of a single disease cause.

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[4] Rous and Lange, *loc. cit.*

That chicken tumors of markedly different type have different filterable agents as their cause has been proved by experiments already reported. The present findings make it probable that, within certain limits, tumors of rather various character may be dependent upon a single agent. This assumption greatly simplifies the etiological problem. But the truth of the assumption for other instances than those described in the present article can only be determined by the study and comparison in many hosts of the disease-complexes of which each spontaneous chicken tumor is to be considered as an individual expression.

EXPLANATION OF PLATES.[5]

PLATE 69.

FIG. 1. Section of the spontaneous tumor No. 38. It is composed of spindle cells in strands, with abundant collagen. There is some round-celled infiltration.

FIG. 2. A solid growth of the third generation of transplants. The heavy black spots are artefacts.

PLATE 70.

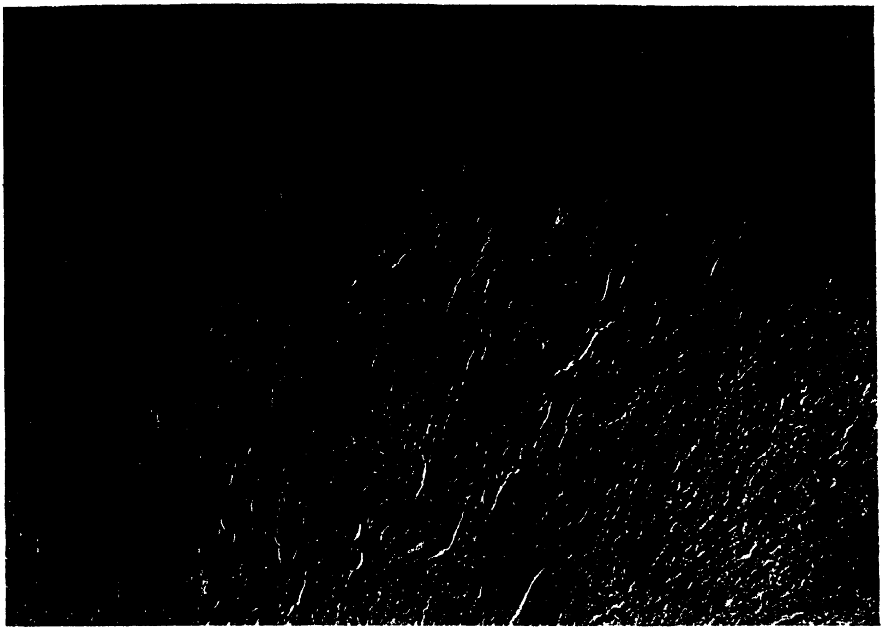
FIG. 3. Portion of a growth that resulted from the injection into a normal fowl of tumor tissue that had been dried while frozen. The rifting with blood sinuses here shown has been found in several transplantation growths as well.

FIG. 4. Spontaneous Tumor 27. Section of one of the growths in the skeletal muscles.

PLATE 71.

FIG. 5. Secondary growths in a fowl of the first transplantation series of Tumor 38. The primary growths were situated in the pectoral muscles. They have been removed with the sternum except for a small portion of that on the left (*A*). There are metastases in both legs near the knee (*B*), in the neck muscles (*C*), and in the muscles within the bony trunk (*D*). A number of nodules coalescing into a thick cord (*E*) connect the junctions of the sternal and vertebral ribs.

[5] The microscopic sections were stained with methylene blue and eosin. The illustrations should be compared with those of Chicken Tumor 18 (Rous and Lange, *loc. cit.*).



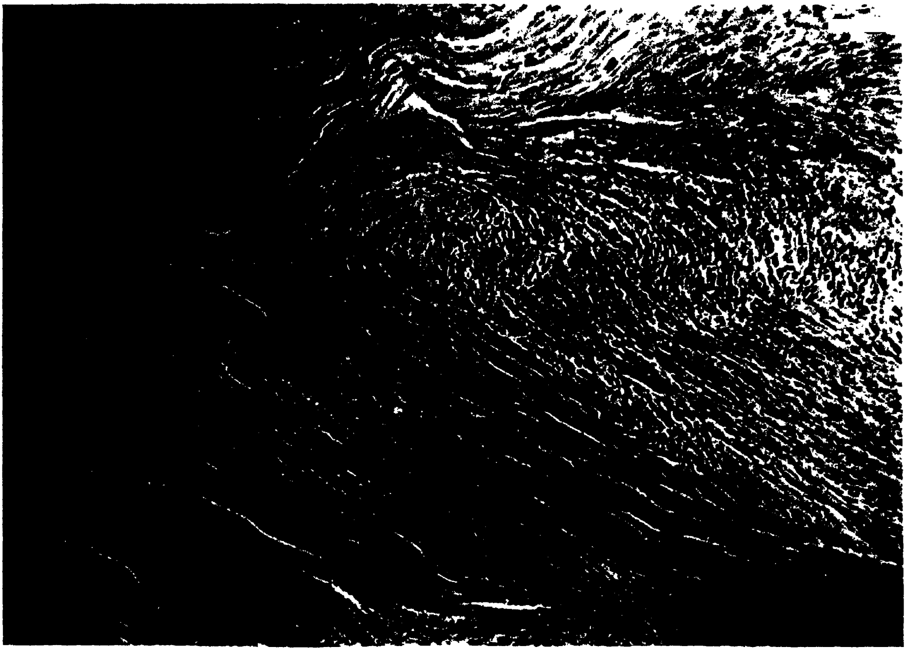


FIG. 2.

(Rous: Spontaneous Chicken Tumors.)

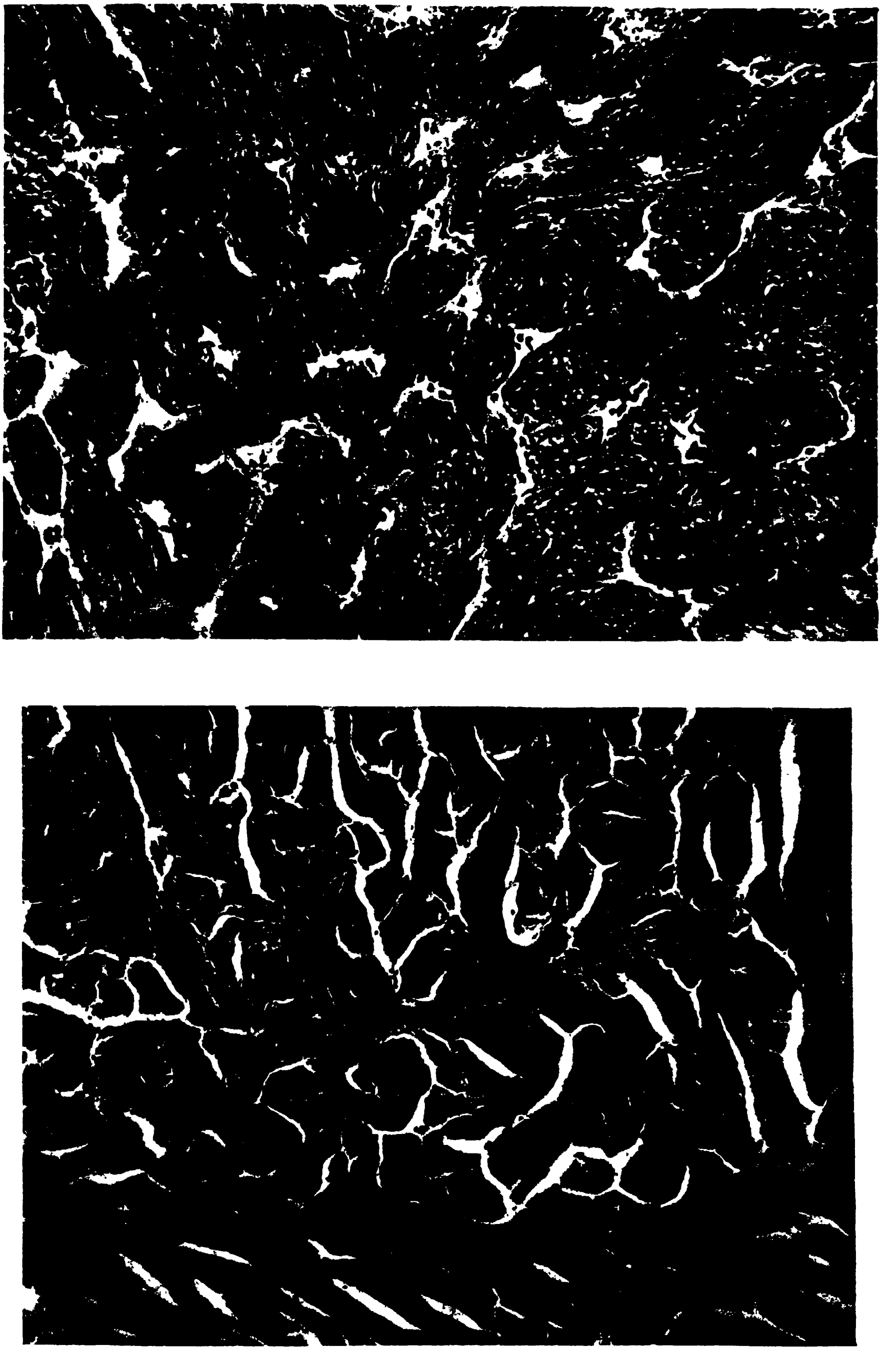


FIG. 4.

(Rous: Spontaneous Chicken Tumors.)

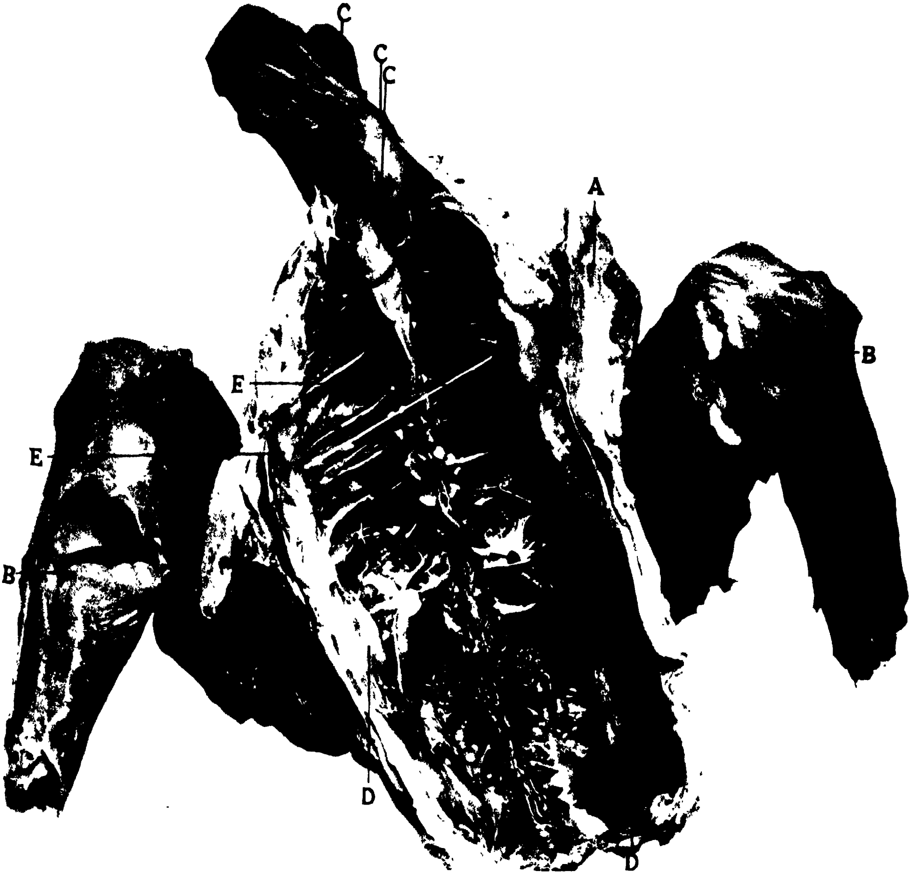


FIG. 5.

(Rous: Spontaneous Chicken Tumors.)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, June 1, 1914, Vol. xix, No. 6, pp. 577–580.]

ON CERTAIN SPONTANEOUS CHICKEN TUMORS AS MANIFESTATIONS OF A SINGLE DISEASE.

II. SIMPLE SPINDLE-CELLED SARCOMATA.*

BY LINDA B. LANGE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 72 TO 74.

Among the spontaneous chicken tumors recently brought to this laboratory there have been two spindle-celled sarcomata that have yielded, on transplantation, neoplasms similar respectively to two strains already under propagation. The resemblance of the growths derived from Chicken Tumor 38 to those derived from Chicken Tumor 18 is taken up in the preceding article.[1] They are spindle-celled sarcomata of protean character, often rifted with blood sinuses in a characteristic manner and showing a tendency to metastasize to the voluntary muscles. The subject of the present paper is Chicken Tumor 43, a simple spindle-celled sarcoma, apparently identical with Chicken Tumor 1.

The spontaneous tumor No. 38 differed considerably from the spontaneous tumor No. 18, and only after the growths had been observed in many hosts was their close similarity realized. Tumor 43, on the other hand, in its original form strikingly suggested Tumor 1, and the transplantation growths are practically identical with those of the latter. Both are produced by a filterable agent.

GROSS CHARACTERISTICS.

The original Chicken Tumor 43 occurred in a Plymouth Rock hen as a large nodular mass in the substance of the pectoral muscle, loosely attached to the lower end of the sternal keel, but not in-

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* Received for publication, April 20, 1914.

[1] Rous, P., *Jour. Exper. Med.*, 1914, xix, 570.

volving the skin. The mass was fairly well defined, but devoid of a capsule. On incision the tumor tissue was, for the greater part, smooth, greyish pink, and firm, traversed by a few large blood sinuses, and well nourished throughout.

Pieces of the fresh tissue were inoculated into the pectoral muscles of two Plymouth Rock fowls by means of small trocars, the method employed also in subsequent transfers. The tumor grew in one of the two fowls. The rate of growth was slow at first, but on transplantation the malignancy increased rapidly, and in the fourth tumor generation the number of takes reached 100 per cent. The rate of growth as measured by the longest diameter was three times as rapid in the eighth as in the first generation. As contrasted with the firm almost gristly tissue of the earlier growths the more malignant tumors of later generations have been translucent and friable, and wet with a mucinous fluid. Hemorrhage into the tumors is frequent. In resistant fowls, on the other hand, the growth is firm, dense, nodular, and may undergo liquefaction resulting in cysts containing a clear mucinous fluid. With the progressive enlargement of the tumors the host emaciates, becomes cyanotic, and finally dies in coma.

Metastases were found in the original fowl in the heart and lungs, but not again until the fourth transplantation generation when they were also situated in the heart and lungs. Metastases have since been fairly frequent. They usually occur in the heart, lungs, and liver, less often in the spleen and kidney (figures 1 and 2). Implantation tumors on the heart and liver from a tumor growing through the body wall have been observed once.

MICROSCOPIC FINDINGS.

The tissue of the original growth is composed for the most part of slender spindle cells of somewhat irregular size, with pale, oval, vesicular nuclei often containing elongated or double nucleoli. Mitotic figures are fairly frequent. Scattered through the tissue are a few giant cells (figure 3). In some areas the cells are plumper and irregularly oval. The tissue structure varies, being very compact in some places and loosely meshed in others.

This picture has been fairly constant. Among the transplantation growths giant cells have been infrequent and the tumors have presented the general characters of a simple, spindle-celled sarcoma. In hosts relatively resistant, as shown by the behavior of the tumor, accumulations of small round cells are found, especially at the edge of the neoplastic tissue. The vigorously growing tumors are composed of spindle cells fairly uniform in size, shape, and arrangement. At the edge of the tumors there is practically no cellular reaction. When very malignant the tumor invades the muscle, not only by growing between the muscle bundles and fibers, but by penetrating the sarcolemma and replacing the muscle substance directly (figure 4). The metastases are histologically identical with the primary tumors.

ETIOLOGY.

Bacteriological cultures from the tumor tissue on the ordinary media have remained sterile under aerobic and anaerobic conditions. A causative agent separable from the tumor cells has, however, been demonstrated. The clear fluid obtained by filtering a thin suspension in Ringer solution of the finely ground tumor through Berkefeld filters holding back a test bacterium at the same filtration is capable of giving rise to tumors in normal fowls. These filtration tumors are identical with those from which the tissue for the emulsion was obtained. Tissue ground, frozen, and dried *in vacuo* over sulphuric acid, made up to the original bulk with distilled water, and injected into normal chickens, likewise causes tumors. Finally, the opalescent fluid obtained by centrifugalizing a thin suspension of the ground tissue in Ringer's solution may be mixed with glycerin and kept at 5° C. for many days without losing its ability to cause tumors. Mixtures containing 80 per cent. glycerin tested after ten days, and those containing 50 per cent. glycerin tested after twenty-one days were still active. Tumors arose from two to three weeks after inoculation of the material. As with the other chicken tumors, no case of cage infection has ever occurred.

RESEMBLANCE TO CHICKEN TUMOR I.

The spontaneous Chicken Tumor 43 and its transplanted growths closely resemble those of the strain known as Chicken Tumor 1.[2] They are of similar gross and microscopic structure, run the same course, metastasize, in general, to the same organs and have the same general action on the host. In appearance and behavior they are, indeed, indistinguishable. An etiological agent distinct from the living cells is easily demonstrated for both tumors by filtration, desiccation, and glycerination. In the absence of definite experiments upon the point it cannot be affirmed that both tumors have the same cause, yet a closer parallelism between two strains of the same disease obtained by transfer from separate instances occurring in nature could hardly be looked for. In this connection it is of interest to note that Chicken Tumor 13 (figure 5), a growth arising in the connective tissue of the foot, has some resemblance in its histology to Nos. 1 and 43, though it may not with certainty be classed with them. The attempt to propagate No. 13 was unsuccessful and nothing can be said as to its etiology.

SUMMARY.

The forty-third spontaneous chicken tumor received at this laboratory strikingly resembles the first and has given rise on transplantation to an entirely similar series of neoplasms. Tumors of both strains are due to a filterable agent which remains active in the dried or glycerinated tissue.

EXPLANATION OF PLATES.[3]

PLATE 72.

FIG. 1. Characteristic tumor filling out the left breast of the fowl. The right breast shows the emaciated state of the fowl. Metastases can be seen in the heart, lungs, and liver, as indicated by the arrows. This tumor arose from glycerinated tissue.

FIG. 2. Metastasis in the heart from a tumor of the eighth transplantation generation. There is a complete absence of a cellular reaction about the growth.

PLATE 73.

FIG. 3. Section of the original growth showing subcutaneous spindle-celled neoplasm with scattered giant cells.

FIG. 4. Tumor of the sixth transplantation generation invading striated muscle. The muscle fibers are directly replaced by tumor cells.

PLATE 74.

FIG. 5. Section of Chicken Tumor 13.

[2] Rous, P., *Jour. Exper. Med.*, 1910, xii, 696; 1911, xiii, 397.

[3] The microscopic sections were stained with methylene blue and eosin.

FIG. 1.

(Lange: Spontaneous Chicken Tumors.)

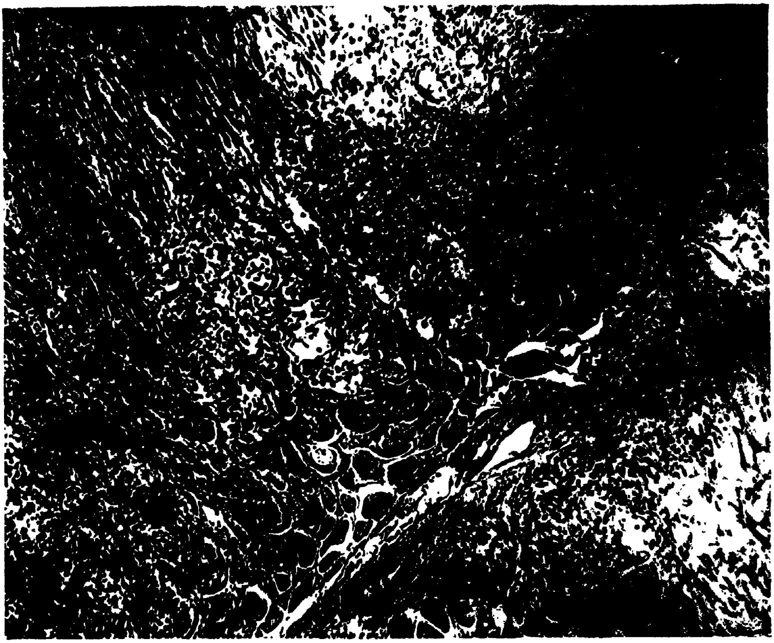

FIG. 2.

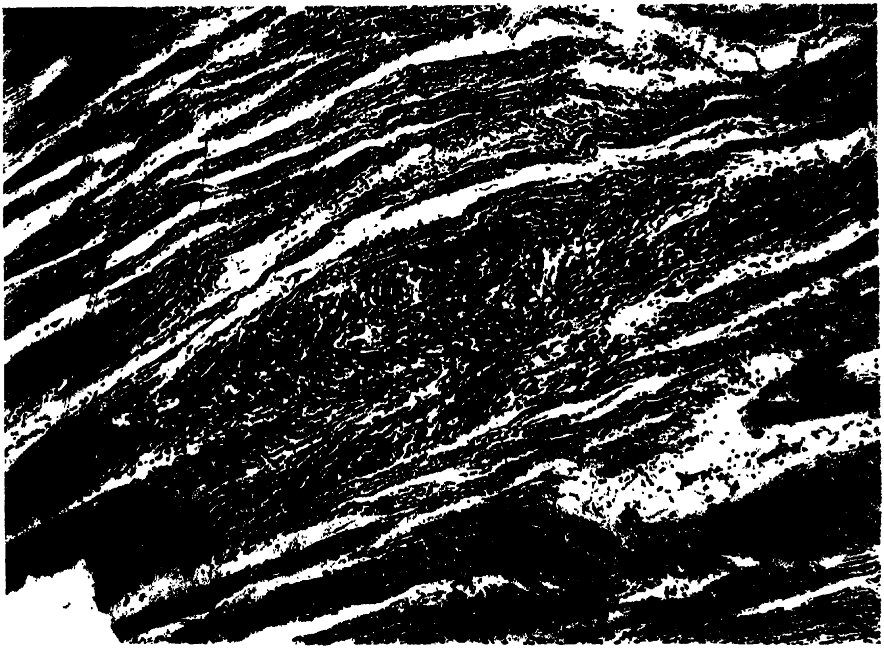

FIG. 3.

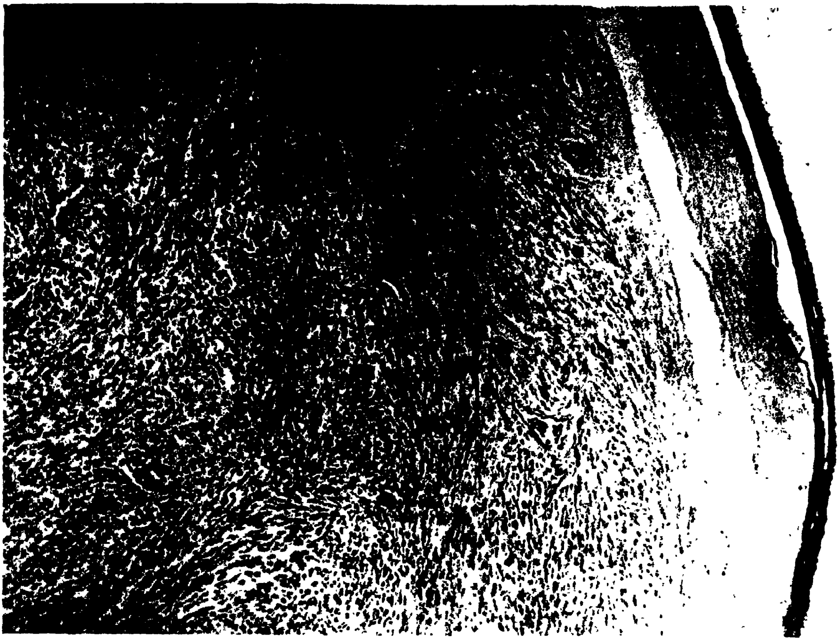

FIG. 4.

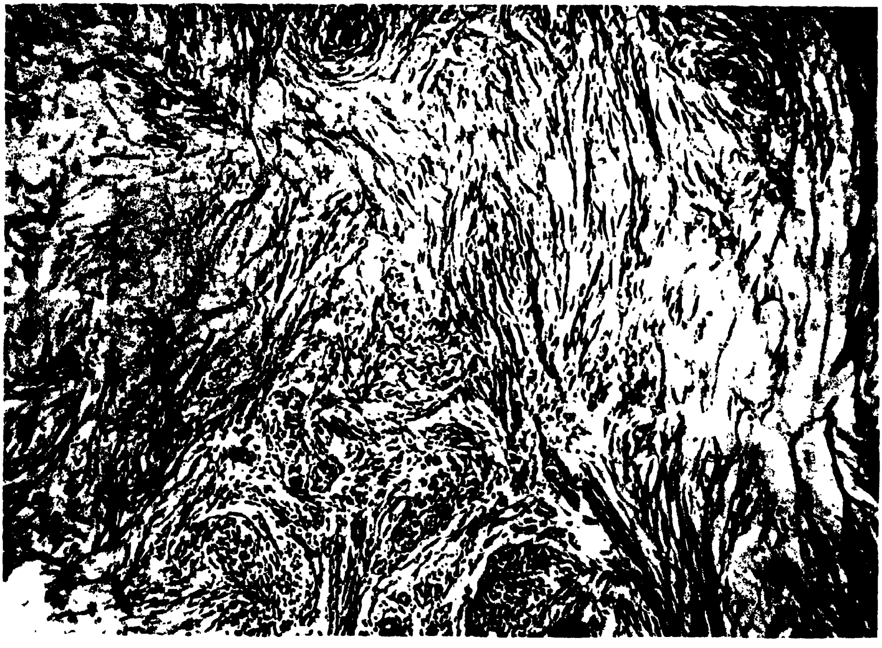

FIG. 5.

(T : Spontaneous Chicken Tumors.)

[Reprinted from The Johns Hopkins Hospital Bulletin, June, 1914, Vol. xxv, No. 280, pp. 173–177.]

THE BLOOD-PICTURE IN HODGKIN'S DISEASE.

Second Paper.[1]

By C. H. BUNTING, M.D.

(From the Pathological Laboratory of the University of Wisconsin, Madison.)

In an earlier paper[2] on Hodgkin's disease, I pointed out characteristics in the blood-picture which seemed at that time to warrant the conclusion that the diagnosis of the disease might be made from blood smears with a considerable degree of accuracy. The earlier paper was based on a study of 11 cases, in all but one of which the diagnosis was established by microscopical examination of a test-gland; and in the one exception, the clinical picture and the course of the disease left no doubt as to the diagnosis.

During the past year it has been possible, through the courtesy of friends in the medical profession, to study the blood of 14 additional cases, in which the diagnosis has been established by the histological examination of a test gland. It has thus been possible to confirm and strengthen the earlier findings.

The series of cases studied includes 15 males and 10 females, a somewhat higher percentage of the latter sex than is usually given. It is further rather striking, that the great majority of the males are under the thirty-third year, while the majority of the females are above that age.

The study of the blood in these cases has shown, as indicated by the tabulated results, that there is a deviation from the normal leukocytic picture in all cases, but that there is not a single constant picture found in them. Instead, it is possible to divide the cases into two distinct groups according to the differential count of the leukocytes. The first group, including cases of a year or less in duration, shows a normal or slightly increased total leukocyte count with a normal or decreased percentage of polymorphonuclear neutrophiles. The second group includes the cases of greater duration for the most part, and shows a sharp leukocytosis, running in one case (as far as could be determined from the smear ratio of 1 white cell to 29 red cells), to at least 100,000 leukocytes per cmm. This leukocytosis is accompanied by an increase of the neutrophiles to a percentage between 72 and 90—a percentage ordinarily considered of value in diagnosing a suppurative process in the body, yet occurring in Hodgkin's disease in the complete absence of pus formation.

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[1] This work has been aided by a grant from The Rockefeller Institute for Medical Research, New York.

[2] Johns Hopkins Hosp. Bull., 1911, XXII, 369.

GROUP ONE.

Case No.	Sex.	Age.	Appar. duration.	Leukocyte count.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
I	M.	21	4 mos.	7,500	51.2	3.2	1.6	25.4	10.	....	8.6
II	M.	10	1 yr.	9,500	56.2	8.6	5.4	16.4	7.4	....	11.
IV	F.	40	9 mos.	....	36.	0.6	0.2	39.2	10.6	2.8	10.6
V	M.	8	5 mos.	9,900	59.4	4.6	0.4	21.8	3.6	....	10.2
VII	F.	64	3 mos.	4,480	54.8	4.2	0.4	20.	9.8	1.6	9.2
IX	M.	5	5 mos.	4,200	54.4	2.4	0.2	22.6	8.6	2.4	9.4
XIII	M.	31	14 mos.	6,000	44.8	4.6	0.4	19.4	13.4	2.4	15.0
XVIII	M.	33	6 mos.	....	64.2	0.4	0.0	16.4	9.2	0.6	9.2
XIX	M.	33	5 mos.	....	46.8	0.6	1.4	33.4	3.8	0.4	13.6
XXI	M.	37	18 mos.	low	70.4*	1.8	0.4	10.6	3.6	0.0	13.2
XXIII	M.	17	?	10,000	60.4	2.	0.6	20.8	6.2	0.2	9.8

* Count taken with recent surgical incision still unhealed.

GROUP TWO.

III	M.	30	*	10,000	79.4	0.2	0.4	5.8	....	3.4	10.8
VI	M.	16	?	18,000	81.6	0.6	0.4	4.2	3.	1.6	8.6
VIII	M.	33	....	....	76.2	1.4	0.6	7.6	....	6.	8.2
X	M.	....	....	14,300	84.	0.0	0.0	3.8	1.0	1.0	10.2
XI	M.	22	1 yr.	....	81.8	0.2	0.6	2.6	2.6	1.0	11.2
XII	F.	33	1 yr.	19,000	85.4	2.8	0.0	3.2	0.4	0.2	8.
XIV	F.	52	2 yrs.	100,000	89.2	0.0	0.0	2.4	1.0	0.6	4.
XV	F.	20	1 yr.	9,000	84.4	1.2	0.0	2.2	4.2	0.4	7.6
XVI	F.	34	†	12,400	85.8	0.2	0.4	4.4	3.2	0.6	5.4
XVII	F.	38	2 yrs.	22,000	82.6	2.	0.0	5.2	2.4	0.0	7.8
XV	F.	24	1 yr.	20,000	90.4	0.4	0.0	2.4	1.4	0.0	5.4
XXII	F.	22	2 yrs.	6,200	78.	2.6	1.2	4.4	0.2	0.0	13.6
XXIV	M.	27	7 mos.	44,000	84.	1.6	0.0	8.8	0.4	0.2	5.
XXV‡	F.	17	4 mos.	12,000	72.6	0.9	0.4	15.3	3.0	0.4	7.4

* Over 1 year. † 10 months. (?)

N. = Neutrophile, E. = Eosinophile, B. = Basophile, S. L. = Small Lymphocyte, L. L. = Large Lymphocyte, L. M. = Large Mononuclear, Tr. = Transitional.

‡ Case XXV was apparently counted in a transition from the early to the late blood-picture. Later counts show an increase in neutrophiles to 78.4% and a decrease in lymphocytes to 7.6%.

The explanation of this change in blood-picture is not entirely clear. While the primary blood-picture is usually found in cases of relatively short duration, and with comparatively localized lesions, there are enough exceptions in the series to indicate that these are not the sole factors. If it were possible to examine every gland in every case one might find a pathological explanation for the change. Glands from two of the cases with the sharpest leukocytosis indicated that in these the disease was progressing more intensely. There was more necrosis and a marked infiltration of the gland and surrounding tissue with neutrophile leukocytes. As a result of the bacteriological investigations of Dr. Yates and myself,[8] I was on the point of accepting the possibility that complicating infection with staphylococci might be responsible for this increased intensity of reaction when cultures from several glands in a very recent case with such a picture gave only the diphtheroid organism. Inoculation of monkeys with the diphtheroid organism has produced the primary blood-picture, with a slight tendency toward the secondary picture in one monkey, inoculated with an organism of increased virulence. Pathological study of these experimental lesions indicates strongly that while necrosis of lymphocytes leads to chemotaxis for eosinophiles, the necrosis of the proliferated endothelioid cells and fibroblasts leads to positive chemotaxis for neutrophiles. Thus, in the cases with greater intensity of the process, we have the neutrophile leukocytosis.

Turning to the other changes in the differential count, the most striking feature, as indicated in the earlier paper, is the increase in the so-called transitional leukocyte—the large mononuclear cell with indented or lobed nucleus, abundant protoplasm and fine azurophile granulation with Wright's stain. These cells are absolutely increased in all cases, and relatively increased in all except those with well marked leukocytosis; and even in these latter cases the transitionals are the most numerous cells except the neutrophiles. Thus, in the group of cases with the primary picture, one finds the percentage varying from 8.6 to 15, as opposed to a normal of 7.5; while in the secondary group, the percentage is above normal excepting in four cases with a relatively sharp leukocytosis, and in these it

[8] Arch. Int. Med., 1913, XII, 236.

varies from 4 to 5.4. There seems every evidence from blood counts and gland sections in a variety of conditions that these cells are derived from the cells of the germinal centers of lymphoid tissue, and from cells lying upon the reticulum of the lymph cords, and not from the endothelial lining of the lymph sinuses.

The lymphocytes, except for a moderate increase during the first few months of the disease (35.4, 37.2, 49.8 per cent), show a gradual percentage diminution, until in the later cases they vary from 7.6 to 3.4. Apparently the toxin active in the disease, in small doses, leads to a stimulation of lymphocyte production, but in large doses leads to their destruction.

The circulating eosinophile cells show quite a variation in number in the different cases. This seems to depend chiefly upon the reactive power of the marrow. The chemotactic substance which attracts the eosinophile to the glands appears to be some product resulting from the destruction of lymphocytes. Even in the early phase, when this destruction is relatively slight, and there is chiefly proliferation of lymphocytes, the normal marrow does not seem able to compensate and there is a circulatory deficiency. Later, in the well established cases, even with greater demands, marrow compensation is usually excessive, and there is a moderate eosinophilia. In exceptional cases there may be an extreme eosinophilia as shown by a blood smear sent me from the medical laboratory of the Johns Hopkins Hospital, in which there was 68 per cent of eosinophiles in a count of 20,000, and as seen in Case XII of this series, in whom on one occasion there was 33 per cent of eosinophiles in a count of 30,000 leukocytes. In the latter case a rather marked skin reaction to the X-ray may have accounted for the increase.

The basophiles are increased in very early cases, but later almost disappear from the circulation. Basophiles, as well as eosinophiles, may be found in smears from the lymph glands in Hodgkin's disease. This, taken with the blood counts in monkeys inoculated with the diphtheroid organism, suggests that the basophile reaction is specific. However, basophiles appear to be constantly increased in chronic nasopharyngeal and nasal sinus infections, and it may be that such infections in conjunction with the primary lesion of the disease, lead to the early increase in Hodgkin's disease.

In all cases the great increase in blood platelets, noted in the earlier paper, has been found, and with the increase there are always abnormally large platelet masses and pseudopodia.

One may summarize the blood finding then as follows: throughout the disease there are two constant features, an increase in blood platelets and an absolute increase in the transitional leukocytes. In regard to the other elements, in early cases there is a transitory increase in lymphocytes and basophiles, and a deficiency in eosinophiles, with a normal or low neutrophile count, followed by a gradual decrease in lymphocytes and a moderate eosinophilia. In late cases there is a marked neutrophile leukocytosis, and a diminution in percentage of all other elements except the transitional leukocyte.

All of these features of the blood-picture in Hodgkin's disease have been reproduced in the monkey following inoculation with the diphtheroid organism isolated from cases of the disease. One finds, as shown by the counts from one monkey here given, the prompt increase in the transitionals and basophiles, the early deficiency in eosinophiles followed by an eosinophilia, and the early stimulation of the lymphocytes, followed by a gradual reduction. The counts are as follows:

Date.	Total count.	N.	E.	B.	S. L.	L. L.	L. M.	Tr.
April 15	22,300	51.0	2.4	0.2	40.8	2.8	0.4	2.4
19	Inoculation							
21	17,600	45.4	1.0	0.8	38.2	5.2	0.6	5.8
24	14,000	28.2	1.6	1.2	56.0	5.2	0.6	7.2
May 1	31,250	62.0	1.8	0.4	27.2	2.2	0.4	6.0
23	20,000	42.8	2.2	0.8	46.0	2.2	1.0	5.0
24	Inoculation							
28	......	34.4	5.0	0.0	49.6	1.8	0.4	8.8
June 7	Inoculation							
11	14,000	29.8	5.4	0.2	54.8	4.6	5.2	7.0
21	Inoculation							
30	......	50.0	1.8	0.0	38.0	2.0	0.2	8.0
30	Inoculation							
July 1	......	51.8	2.2	0.2	37.2	2.0	0.2	6.4
2	......	48.6	3.8	0.2	36.2	3.6	0.4	7.2
3	......	51.2	1.6	0.2	35.	3.2	0.2	8.6
5	24,000	52.8	2.4	0.0	32.	4.6	0.4	7.8

A gland, removed after the last count recorded, showed many mitoses in endothelioid cells, apparently accounting for the circulatory increase in transitionals. There was also well-marked eosinophilic infiltration, and a lessened production of lymphocytes.

From the foregoing it may be seen that there are sharply marked blood changes in Hodgkin's disease. The question naturally arises: are they of value in diagnosis of doubtful cases? It is my belief that given a case with chronic glandular enlargement and without any suppurative process, and the blood-picture which I have designated as the late or secondary, the diagnosis is established. Given the chronic glandular enlargement and the primary blood picture, diagnosis is somewhat more difficult, but, in my experience, can in the great majority of cases be made with certainty. The diagnosis usually lies between Hodgkin's disease and tuberculosis. There are apparently two distinct pictures in tuberculosis of the glands—that found before there is any softening and abscess formation in the glands, and that found when such a change has occurred. The primary picture here is quite distinctive; the secondary picture is more like the early Hodgkin's picture, but the clinical examination in such a case would determine the abscess formation.

The blood counts which I have made in cases of tuberculosis of the glands are as follows:

CASES WITHOUT ABSCESS FORMATION.

Cases.	Total count.	N.	E.	B.	S. L.	L. L.	L. M.	Tr.
I	....	56.	1.8	0.2	26.4	5.	3.	7.6
II	....	64.8	1.8	0.2	19.4	6.	1.	6.4
III	7,200	59.8	0.2	0.2	31.	1.4	0.0	7.4
IV	10,000	58.	0.6	0.2	33.	2.2	0.0	6.0
V	....	61.2	0.6	0.2	28.6	1.8	0.8	6.0
VI	5,000	46.4	1.4	0.2	45.2	1.4	0.0	5.4
VII	....	61.6	1.2	0.2	20.2	9.	0.6	7.2
VIII	9,400	57.6	1.2	0.6	28.	7.4	0.4	4.8

CASES WITH ABSCESS FORMATION.

I	9,000	41.6	2.4	0.0	43.2	4.4	0.4	8.
II	....	63.	1.2	0.6	19.	6.	2.2	8.
III	11,000	68.6	3.6	0.2	12.4	6.6	0.6	7.6
IV	....	53.4	4.2	0.6	26.	7.6	0.2	8.
V	10,800	64.6	5.4	0.2	21.6	0.8	0.8	6.6

The quite constant low eosinophile count associated with a low transitional count in the primary group of tuberculosis cases is in definite contrast to the picture in Hodgkin's disease. In addition, while platelets are usually increased in tuberculosis, one seldom finds so great an increase as in Hodgkin's disease, and the abnormally large platelet masses are, in my experience, lacking in the tuberculosis picture. With the exercise of judgment, therefore, the diagnosis may be made from the blood smear.

One case has thrown some doubt on the possibility of diagnosing between Hodgkin's disease and some forms of the so-called malignant lymphomata. In this case, with a primary orbital tumor of slight greenish cast, and general glandular and splenic enlargement, the blood-picture could not be distinguished from that of Hodgkin's disease. Two counts in that case made at an interval of nine months are as follows:

N.	E.	B.	S.L.	L.L.	L.M.	Tr.
54.6	0.9	0.9	23.3	8.2	0.4	11.5
61.4	0.4	0.8	20.6	3.2	0.4	13.2

This finding may not invalidate the blood-picture in Hodgkin's disease. It may, when taken with the fact that there was isolated from this case an organism similar to, if not identical with, that obtained constantly in Hodgkin's disease, explain the difficulty of morphologists in drawing a sharp line between the various forms of apparently malignant glandular hyperplasia.

Abstracts of the Hodgkin's disease cases, upon which this report is based, follow:

Case I.—(Dr. Yates.) November 20, 1908. Male, white, 21. Onset of disease, July, 1908. Cervical and axillary glands involved. Test gland removed October 30, 1908, showed early Hodgkin's changes. Gradual improvement under treatment. Clinically well since June, 1909, except for some nasal infection.

BLOOD COUNTS.

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
XI. 20, '08	5,304,000	7,500	51.2	3.2	1.6	25.4	10.		8.6
III. 4, '09	4,968,000	4,800	55.2	2.4	0.8	23.6	6.6		11.4
VI. 11, '09	5,600,000	7,000	65.8	2.8	0.0	16.8	7.6	1.2	5.8
II. 16, '10	5,800,000	7,000	59.8	4.0	1.4	15.4	8.8	1.8	8.8
VI. 9, '11	....	8,500	65.0	2.6	1.2	21.2	3.	0.6	6.4
IV. 17, '12	....	5,000	42.2	5.8	0.6	41.4	1.8	0.4	7.8
XI. 17, '13	....	10,000	60.	2.6	0.8	27.4	3.8	0.2	5.2

Case II.—(Dr. Yates.) Male, white, 10. Large mass of discrete glands in left cervical region of at least one year's duration previous to removal, November 10, 1908. Glands show well-marked Hodgkin's picture. Von Pirquet reaction negative October 8, 1910. Gradual improvement under treatment. Increase in size of one gland left at first operation in March, 1913. Removed. Apparently well—1914.

BLOOD COUNTS.

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
IV. 1, '09	4,712,000	9,500	56.2	8.6	0.4	16.4	7.4		11.0
I. 22, '10	5,250,000	9,600	63.6	3.6	0.0	12.0	12.0	2.0	6.8
III. 12, '10	....	....	53.4	5.2	1.0	13.	16.	1.6	9.8
X. 8, '10	4,960,000	9,800	53.2	5.2	0.4	27.8	4.	1.4	8.0
II. 13, '12	....	8,000	57.	4.4	0.2	27.4	4.	0.2	6.8
VI. 3, '12	5,500,000	7,000	64.	4.	0.2	19.8	3.2	0.2	8.4
III. 10, '13	....	....	39.6	10.4	0.6	25.	10.4	1.0	13.0
IX. 18, '13	....	....	64.2	3.6	0.6	22.	1.8	0.0	7.8

NOTE.—Recurrence found after paper was in press, associated with return of blood-picture to that noted earlier during active stage of the disease.

Case III.—April 1, 1909. Male, white, 30. Seen 2 weeks after second operation for glands of neck. First operation 6 months previous at Rochester. Diagnosis, Hodgkin's disease.

BLOOD COUNT.

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
IV. 1, '09	4,560,000	10,000	79.4	0.2	0.4	5.8	3.4		10.8

Case IV.—(Dr. Tupper, Eau Claire, Wis.) February 5, 1910. Female, white, 40. Onset, — months previous in right inguinal glands. Subsequent involvement of left inguinal, left axillary, left and right cervical glands. Test gland shows well-marked active Hodgkin's picture. Death in October, 1910.

BLOOD COUNT.

Date.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
II. 5, '10	36	0.6	0.2	39.2	10.6	2.8	10.6

Case V.—(Dr. Yates.) November 9, 1909. Male, white, 8 years. Enlargement of cervical glands began 5 months previous to date. Left inguinal glands also enlarged. Test gland, November 30, 1909, shows hyperplastic stage of lesion. January, 1910, cervical glands removed. October, 1910, von Pirquet test negative. April, 1911, mucous membrane hæmorrhages, anæmia. Died January 10, 1912. Involvement of mesenteric glands, chylous ascites, wasting.

BLOOD COUNTS.

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr
XI. 9, '09	....	9,900	59.4	4.6	0.4	21.8	3.6		10.2
XI. 30, '09	....	....	50.8	5.8	0.6	26.8	7.2		8.8
II. 26, '10	5,000,000	5,000	55.2	1.4	0.4	22.4	9.2	2.0	9.4
IX. 7, '10	5,160,000	8,700	59.4	1.	1.2	16.4	6.6	5.	10.4
IV. 21, '11	3,456,000	4,800	63.2	1.8	1.	10.2	4.6	1.4	17.8

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
Transfused.									
IV. 24, '11	3,176,000	3,800	54.	6.8	0.2	17.8	4.8	1.8	14.6
V. 2, '12	2,624,000	8,300	67.6	1.4	1.4	6.8	9.6	0.6	12.6
Transfused.									
V. 5, '11	2,760,000	4,500	54.6	2.6	2.	18.8	7.6	0.6	13.8
V. 9, '11	3,200,000	4,000	50.8	4.	1.6	25.8	6.4	0.6	10.8
Transfused.									
V. 10, '11	....	....	60.	9.8	0.6	8.2	6.8	0.4	14.2
V. 13, '11	3,400,000	3,500	56.2	4.8	0.8	17.6	6.4	0.4	13.8
V. 22, '11	3,712,000	3,400	55.8	3.2	0.0	19.8	3.6	1.2	16.4
V. 29, '11	3,680,000	3,600	56.4	3.4	1.6	18.	8.1	0.6	11.8

Case VI.—(Dr. Sullivan, Madison, Wis.) March 1, 1910. Male, white, 16. Left cervical glands much enlarged. Marked induration of neck. Enlargement noted for only 1 month, but the amount of sclerosis in test gland suggests greater duration. March 10, wound resulting from removal of gland for diagnosis still unhealed. May 13, wound healed but induration of neck marked. Death during 1911.

BLOOD COUNTS.

Date.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
III. 10, '10	18,000	81.6	0.6	0.4	4.2	3.	1.6	8.6
V. 13, '10	27,000	80.6	1.6	0.0	7.8	2.8	0.8	6.4

Case VII.—(Dr. Bennett, Oregon, Wis.) January 5, 1910. Female, white, 64. In October, 1909, general glandular enlargement with clinical diagnosis of Hodgkin's disease. Death June 30, 1910.

BLOOD COUNTS.

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
X. 24 '09	3,200,000	4,480							
I. 5, '10	....	....	54.8	4.2	0.4	20.0	9.8	1.6	9.2

Case VIII.—(Dr. Yates.) Male, white, 33. Onset in March, 1907, with supraclavicular glandular enlargement. In September, 1908, supraclavicular and left axillary glands most enlarged. Some enlargement of right cervical, axillary and inguinal glands. October 30, 1908, excised gland shows typical and advanced Hodgkin's disease. Death occurred May 20, 1909.

BLOOD COUNT.

Date.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
I. 10, '09	76.2	1.4	0.6	7.6	6.0		8.2

Case IX.—(Dr. Yates.) October 3, 1910. Male, white, 5. Marked enlargement of left cervical glands of 5 months duration. Test gland shows well marked Hodgkin's changes. Death from shock at operation.

BLOOD COUNT.

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
X. 3, '10	5,160,000	4,200	54.4	2.4	0.2	22.6	8.6	2.4	9.4

Case X.—(Dr. Yates.) Male, white, cervical Hodgkin's. Count made 2 months previous to death of patient.

Date.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
IV. 30, '10	14,300	84.	0.0	0.0	3.8	1.0	1.0	10.2

Case XI.—(Dr. Baird, Eau Claire, Wis.) December 8, 1910. Male, white, 22. Enlargement of supraclavicular gland noted one year previous. Axillary glands enlarged at date. Test gland shows definite Hodgkin's picture.

BLOOD COUNT.

Date.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
XII. 8, '10	81.8	0.2	0.6	2.6	2.6	1.	11.2

Case XII.—(Dr. Yates.) Female, white, 33. October, 1911. Enlargement of the right cervical and axillary glands of one year's duration. Test gland showed a picture of advanced typical Hodgkin's disease. Cervical glands removed February 13, 1912. Subsequent X-ray treatment. Removal of recurrences in right axilla and left sub-clavicular region January, 1913; March, 1913; July, 1913. Clinically well 1914.

BLOOD COUNTS.

Date.	W. b. c.	N.	E.	B.	S. L.	L. L.	L. M.	Tr.
IX. 27, '11	19,000	85.4	2.8	0.0	3.2	0.4	0.2	8.0
X. 3, '11	21,000	72.4	11.0	0.2	5.6	2.2	0.2	8.4
X. 31, '11		73.8	6.2	0.8	7.6	2.5	0.6	8.2
XII. 1, '11	35,000	84.	2.9	1.1	4.7	0.9	0.1	6.3
XII. 29, '11	21,000	72.	11.0	0.4	9.2	0.9	0.0	5.5
II. 13, '12		74.2	9.2	0.2	9.2	1.2	0.0	6.0
III. 15, '12	30,000	48.0	36.	1.4	9.0	1.2	0.0	4.4
IV. 10, '12	17,000	73.2	10.	0.6	7.2	0.6	0.0	8.4
V. 3, '12	17,000	74.2	9.6	0.2	10.8	1.4	0.0	3.6
VI. 1, '12	12,500	77.8	7.4	0.8	8.8	0.0	0.2	5.0
X. 8, '12	16,000	70.	12.6	0.0	8.6	1.8	0.2	7.0
XI. 27, '12	15,000	72.6	8.6	0.4	7.4	3.4	0.0	7.6
I. 11, '13	16,000	80.3	5.0	0.1	4.2	2.6	0.2	7.6
II. 14, '13	13,000	71.8	12.2	1.0	7.4	1.4	0.0	6.2
III. 3, '13	15,500	64.8	13.2	0.6	12.4	3.2	0.0	5.8
IV. 12, '13	15,000	74.	6.8	1.0	10.0	0.8	0.0	7.4
VII. 14, '13	15,000	71.	8.8	1.8	10.6	1.6	0.0	6.2
VIII. 4, '13	7,000	51.0	10.0	0.0	27.0	0.8	0.2	11.0
I. 22, '14	11,000	66.0	9.6	0.8	15.0	1.4	0.6	6.6

Case XIII.—(Dr. L. F. Barker, Baltimore.) Male, white, 31. Enlargement of cervical glands, right side, of fourteen months duration. Test gland positive.

BLOOD COUNT.

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
XII. 23, '12	5,700,000	6,000	44.8	4.6	0.4	19.4	13.4	2.4	15.0

Case XIV.—(Dr. S. T. Reeves, Albany, Wis.) Female, white, 50. The glandular swelling had first appeared in the left cervical region two months previous to the patient's death. At the post mortem examination January 15, 1913, there was found rather general glandular involvement, but especial enlargement of the mesenteric and retroperitoneal nodes and of the lymphoid elements of the spleen. There was also a chylous ascites, well marked anemia and emaciation. Leukocyte count estimated from ratio of 1 white cell to 29 red cells.

BLOOD COUNT.

Date.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
XII. 27, '12	100,000	89.2	0.0	0.0	2.4	1.0	0.6	4.0

Case XV.—(Cook County Hospital, Chicago.) Female, white, 20. Marked enlargement of cervical glands; moderate of axillary and inguinal glands. Spleen palpable. Duration fourteen months. Test gland positive.

BLOOD COUNT.

Date.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
II. 18, '12	9,000	84.4	1.2	0.0	2.2	4.2	0.4	7.6

Case XVI.—(Drs. Mayo, Rochester.) Female, white, 34. Four years previously she noticed a swelling on the right side of her neck, followed by the appearance of other small tumors. Ten months before date enlarged lymph-nodes appeared on the left side of the neck and in the right axilla. Six weeks previously nodes appeared in the left axilla. Physical examination revealed, in addition to these nodules, a mediastinal mass, and a six months' pregnancy, over which were felt discrete firm nodules which slipped under the examining hand. Test gland positive.

BLOOD COUNT.

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
I. 9, '13	4,480,000	12,400	85.8	0.2	0.4	4.4	3.2	0.6	5.4

Case XVII.—(Drs. Mayo, Rochester.) Female, white, 28. Marked enlargement of the cervical nodes and rather general glandular enlargement. The illness began with involvement of the cervical glands two years previous to date. Test gland showed extreme sclerosis with many giant cells and eosinophiles in the meshes.

BLOOD COUNT.

Date.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
II. 15, '13	22,000	82.6	2.0	0.0	5.2	2.4	0.0	7.8

Case XVIII.—(Drs. Mayo, Rochester.) Male, white, 33. The patient had

had an abscess of a tooth six months previously. About three weeks before coming to the hospital patient had noticed a painless swollen node in the left supraclavicular region. This had increased in size during the time of observation. The large node removed was of a uniform medullary appearance on gross section, and microscopically showed lymphoid and endothelial hyperplasia, with the presence of giant cells, beginning diffuse sclerosis and eosinophilic infiltration. The architecture of the node was destroyed.

BLOOD COUNT.

Date.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
I. 31, '13	64.2	0.4	0.0	16.4	9.2	0.6	9.2

Case XIX.—(Dr. Frank Billings, Chicago.) Male, white, 32. Loss of weight since January 1, 1913. Glandular enlargement noted in February, 1913 On examination, in April, moderate enlargement of cervical, axillary and inguinal glands, tonsils and spleen. Abscess of right upper molar found. Gland showed early changes.

BLOOD COUNTS.

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
IV. 28, '13	4,150,000	4,150							
V. 29, '13	....	....	46.8	0.6	1.4	33.4	3.8	0.4	13.6

Case XX.—(Dr. C. P. Howard, Iowa City.) Female, white, 24. January, 1913. Enlargement of glands of left axilla noted. In February, 1913, glands of right axilla enlarged; also those of both sides of the neck and of the mediastinum. Gland positive.

BLOOD COUNT.

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
XII. 8, '13	4,780,000	20,000	90.4	0.4	0.0	2.4	1.4	0.0	5.4

Case XXI.—(Dr. Yates.) Male, white, 37. Cervical glandular enlargement for twenty-one months. Removal of single glands at various times. Tonsillectomy June, 1913. December, 1913, large group of glands in left side of neck; no other enlargement.

BLOOD COUNT.

Date.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
XII. 13, '13	Low	70.4	1.8	0.4	10.6	3.6	0.0	13.2

Case XXII.—(Dr. G. E. Pfahler, Philadelphia.) Female, white, 22. Cervical glands noted in November, 1911. Subsequent involvement of mediastinal, mesenteric and retroperitoneal glands.

BLOOD COUNT.

Date.	R.b.c.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
XII. 10, '13	4,420,000	6,200	78.0	2.6	1.2	4.4	0.2	0.0	13.6

Case XXIII.—(Dr. H. L. Ulrich, Minneapolis.) Male, white, 17. Operation for glands of the neck at age of 10. Operation at Rochester, November, 1912, for glands of the neck. Diagnosis: tuberculosis. Gland removed in 1913; diagnosed at University of Minnesota laboratory as Hodgkin's disease. In December, 1913, enlarged glands in both sides of neck and in right axilla. Test gland shows unmistakable, typical well-developed Hodgkin's disease lesion.

BLOOD COUNT.

Date.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
XII. 29, '13	10,000	60.4	2.0	0.6	20.8	6.2	0.2	9.8

Case XXIV.—(Dr. Yates.) Male, white, 27. Glandular enlargement first noted in May, 1913. Marked enlargement of cervical axillary and mediastinal glands. Marked periglandular inflammation. Test gland shows intense and relatively acute reaction; but typical Hodgkin's disease features.

BLOOD COUNT.

Date.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
XII. 20, '13	44,000	84.	1.6	0.0	8.8	0.4	0.2	5.0

Case XXV.—(Dr. Yates.) Female, white, 17. Glandular enlargement first noted in September, 1913. Marked involvement of left cervical and axillary regions and of mediastinum. Well-marked periglandular reaction. Sclerosis of excised gland indicates greater duration.

BLOOD COUNT.

Date.	W.b.c.	N.	E.	B.	S.L.	L.L.	L.M.	Tr.
I. 17, '14	15,000	72.6	0.9	0.4	15.3	3.0	0.4	7.4

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HODGKIN'S DISEASE.[1]

BY C. H. BUNTING, M.D.

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My interest in Hodgkin's disease dates from a series of experiments performed in the laboratory of Dr. Flexner at the University of Pennsylvania in 1903. In some of these experiments, the intraperitoneal injection of a lymphotoxic serum produced in the mesenteric lymph glands of the rabbits changes which were of the nature of the earliest changes in Hodgkin's disease; that is, there was necrosis of lymphocytes, proliferation of endothelioid cells, infiltration by eosinophiles and a proliferation of fibroblasts independent of the gross framework of the gland.

These results, coupled with the inability of those working on the disease at that time to find pathogenic organisms in the glands, led to the development of a working theory as to the pathogenesis of Hodgkin's disease which I still hold, though in a modified form. This conception of the disease was, that the changes in the lymph glands were due to the filtration through them of a toxin elaborated at some primary focus of infection, and were in consequence entirely of a secondary nature—an end-result. For the explanation of the progress of the disease, I must introduce a subsidiary theory which seems borne out by pathological and experimental experience, that lymphadenoid tissue affords not only a mechanical, but also a chemical, filter for peripheral lymph, protecting the body cells generally and the red blood cells in particular, from a variety of toxins, at the expense of its lymphocytes. So in Hodgkin's disease, the primary group of glands, for a considerable length of time, protects the body from the toxin elaborated by the infectious agent.

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[1] Paper read at a meeting of The Johns Hopkins Hospital Medical Society, Feb. 2, 1914. The work forming the basis of this report has been aided by a grant from The Rockefeller Institute for Medical Research.

The removal of that group of glands by its ultimate sclerosis or by the hand of the surgeon, while the primary focus remains intact, exposes the body to an extension of the process to further glandular groups and, eventually, to an anæmia and a cachexia.

This theory slumbered until 1908, when, on my return to Wisconsin, I found that Dr. Yates was deeply interested in the disease, and we decided to join forces in an attempt to unravel its difficulties and to further its treatment. We have been handicapped throughout by the distance between us, and more by our inability to control a sufficient material for study. We have, however, through the kindness of friends in the medical profession, come in contact in one way or another with 28 cases.

From the standpoint of general etiology, the most striking feature of this series is that 13 of the cases were females and 15 males; and further, that while the males were, with but a single exception, under 34 years of age (and the exception but 35 at onset), the ages of 8 of the females ranged from 33 to 64 years. Both of these features are in contrast to Ziegler's[2] statistics derived from 210 cases gathered from literature. While it is dangerous to draw conclusions from a small series of cases, it would appear equally dangerous to follow Ziegler's method, if I may judge from a single case of which I have knowledge,[3] included in his series.

The study of these cases from a pathological standpoint has but strengthened the conviction that the lesion of Hodgkin's disease is essentially of inflammatory nature. The work of Reed and of Longcope leaves practically nothing of importance to be added to the description of the changes within the glands themselves, unless one emphasizes the fact that there are cases in which the disease runs so intense a course that the necrosis and the inflammatory reaction in the glands almost overshadow the more usually accepted chronic Hodgkin's disease picture. This appears to occur without secondary infection as far as cultures can determine.

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[2] Die Hodgkinsche Krankheit, Jena, 1911.

[3] Among his cases of Hodgkin's disease of the spleen is included a metastasizing sarcoma of the spleen-pulp, published by me some years ago. In this case there was no involvement of the lymphoid tissue in the spleen or elsewhere.

Aside from the gland lesions there are, however, two points in the pathology of the disease which deserve mention. Careful search will, in the majority of cases, reveal a primary inflammatory lesion which was present before the enlargement of the glands occurred. In primary cervical Hodgkin's disease, this is most apt to be in the tonsils, teeth or nasal sinuses. In one primary cervical case, an otitis media of some duration proved the source of the infection. In a primary inguinal case the physician gave a history of a sharp attack of cystitis preceding the glandular enlargement. However, infection through the gastrointestinal mucous membrane is possible, as shown in cases in which the lesions are confined to the lymphoid tissue of the tract and the mesenteric glands.

The second point I wish to emphasize is that, while the glands in Hodgkin's disease remain discrete, they are, nevertheless, bound together in chronic cases by a mass of sclerotic tissue. In other words, there is in the disease an extraglandular inflammatory process which is of a relatively acute nature in early cases, showing a marked inflammatory œdema and a moderate and diffuse infiltration with polymorphonuclears, as well as with large and small mononuclears. There is an early stimulation of fibroblasts leading to the marked sclerosis of chronic cases.

That there is a general systemic reaction in Hodgkin's disease and not simply a process within a gland or group of glands is indicated by the changes in the blood-picture, to which I have given considerable study, but to which I shall refer only briefly here, as I have considered them in detail in another paper.

A study of 25 cases shows that they may be divided into two groups according to their blood-picture. The first of these groups includes roughly those cases of less than a year's duration, and the second, those of over a year's duration, although duration does not appear to be the chief factor in determining the blood-picture. The blood in both groups shows a marked increase in blood platelets, with the presence of large platelet masses and megalokaryocyte pseudopodia. The only other constant feature is an absolute and usually a relative increase in the so-called transitional cell—the large mononuclear with indented or lobed nucleus and abundant protoplasm with azurophile granulation. This is Mallory's endothelial leukocyte, but I believe it is derived from the cells of the reticulum of lymphoid tissue rather than from the lining of the sinuses.

Except in these two points, the two groups of cases vary in blood-picture. The first group shows little, if any, increase in the total leukocyte count; the second, usually a pronounced leukocytosis, even up to 100,000 cells per cmm. In the very early cases of the first group, there is a deficiency in eosinophiles, a slight increase in basophiles, and a fairly well marked lymphocytosis. This is followed after compensation by a slight eosinophilia, and by a gradual decrease in lymphocytes.

In the second group of cases, we have a neutrophile leukocytosis, the percentage of these cells ranging from 76 to 90 in the series. Lymphocytes are exceeded in number by the transitionals in 10 out of 13 cases falling in this group. Those three may be the only varieties of cells found. The blood-picture appears to be of diagnostic value in cases with chronic glandular enlargement; at least, the differentiation from tuberculosis of the glands is possible. I have not been able, however, to differentiate Hodgkin's disease from certain cases of the so-called malignant lymphoma by the blood smear.

In spite of the failure of others to obtain organisms in cultures from cases of Hodgkin's disease, Dr. Yates and I made efforts, wherever opportunity offered, to find by the cultural method the agent producing the disease. Our early efforts at operation and post mortem examination were failures. Our success in obtaining the organism, which we are convinced is the cause of the disease, was, I believe, the result of the following factors: Choice of a suitable medium, the implantation of large pieces of gland tissue with interglandular tissue, and incubation for a sufficiently long period. These steps were not adopted purely by accident. We felt that the organism was one of the so-called higher bacteria, if not a fungus, and consequently, selected cultural material suitable for the growth of the tubercle bacillus; *i. e.,* Dorsett's egg medium and glycerine-phosphate-agar. We further concluded that the organisms were few in number in the glands and were also difficult to grow, so slices of gland as large as the diameter of the test tube would permit were implanted, with the idea that in this way we should stand more chance of implanting organisms, and also with the thought of furnishing sufficient human proteid to give the organism a start at growth. Finally we sealed the tubes and incubated them indefinitely.

Our first successful implantation was made in February, 1912, when, after 10 days' incubation of tissue from a case, we found growth of a diphtheroid organism in 3 of 6 tubes and on both egg medium and glycerine-phosphate-agar. Since then we have not failed to find the organism in any active case of Hodgkin's disease (untreated by the X-ray), in which we have had opportunity to get cultures. It has been found by us, usually in pure culture, in cervical, axillary, and inguinal glands, and in the spleen. However, not infrequently in some tube a growth of a white coccus would be found, although every effort was made to prevent contamination from the skin of the patient operated upon.

The cultural method indicates that even in relatively acute cases the number of organisms in the glands is small. Growth may appear in but 3 or 4 tubes out of a dozen, and in any one tube there are but few colonies.

The organism grows feebly from most cases when first isolated, and may require careful nursing in order to secure a growth upon the medium independent of the human tissue. Two cultures, however, grew very luxuriantly from the start, and one of these we have used for our experimental work. Growth of this culture, however, was as difficult to obtain, after passage through the monkey, as in the majority of human cases.

The organism is Gram-staining and non-acid-fast, and its most striking feature is its pleomorphism. This is so remarkable that about six weeks were spent in an effort to separate supposed contaminations of the culture. The cultures on different media and at different ages show long, banded, and granular rods, fusiform rods, club shaped and large spherical involution forms, short, plump bacilli with polar staining, and coccoid forms. In the older cultures the coccoid forms predominate. Yet a fresh transfer of such coccoid forms will give, on suitable media, the characteristic, long, diphtheroid bacilli.

While one might not expect that an organism which did not produce death in human beings acutely, would be highly virulent to laboratory animals, yet our early experiments were disappointing. Large doses of 24-hour cultures appeared almost innocuous to guinea pigs and white rats. When recourse was had to the monkey, a similar disappointment was met with. By repeated injections, changes were produced in the lymph glands similar to those found in the glands in early cases of Hodgkin's disease in man. Yet the organism would not gain a foothold, apparently, and the glandular enlargement and the periglandular induration would subside. However, that difficulty was overcome. The first step was the recovery of the organism in pure culture from an abscess developing at the site of an inoculation two days before. This organism was then used for injection of the other monkeys. From one of these monkeys an enlarged gland was removed one week after inoculation in its vicinity, and was implanted subcutaneously into another monkey. This animal died in 10 weeks with marked involvement of its lymphoid tissue. The organism was recovered in pure culture and a small dose was inoculated into a monkey which had proved refractory to the original culture. This monkey died also, within 10 weeks, and with most marked lesions of its lymphoid tissue throughout.

One monkey finally became susceptible to the original culture and has shown progressive glandular enlargement since the last injection in August, 1913. Thus we have demonstrated the pathogenicity of the culture, but its virulence toward the monkey has been increased to such a point that the resulting lesions are of a very acute nature.

The elements of the Hodgkin's disease gland picture are present. One finds the marked proliferation of endothelioid cells, with endothelioid giant-cells, eosinophile infiltration, and a proliferation of fibroblasts. However, these changes are overshadowed by the extensive necrosis of tissue, and the polymorphonuclear leukocyte infiltration of the necrotic areas. These areas appear to be made up chiefly of the newly proliferated endothelioid cells. While this picture may seem quite different from that seen in the more chronic cases in human beings, we have recently had its counterpart in a patient with Hodgkin's disease in whom the course of the disease was of a relatively acute nature.

I have been impressed, also, by the fact that in the monkeys inoculated with the organism of increased virulence, the pathological changes in the lymphoid tissue are in many features strikingly like those described by Councilman, Mallory and Pearce in human cases dying of diphtheria. As a result of these experimental studies, and of the incomplete observation of certain clinical cases, I believe we shall find that some obscure infections in man resulting in extensive subcutaneous inflammatory reaction without pus formation, and in intense glandular reaction, will be proved, upon bacteriological investigation, to be cases of acute Hodgkin's disease.

There is one other relationship of Hodgkin's disease that needs mention. Morphologically there is great difficulty in diagnosing between various types of so-called malignant lymph gland lesions. As I have mentioned earlier, in a case of malignant lymphoma, with a primary, slightly greenish tumor in the orbit, and with general glandular and splenic enlargement, the blood-picture was identical with that in Hodgkin's disease. Furthermore, a culture was obtained from the glands in this case of an organism quite similar to, if not identical with, that obtained from cases of Hodgkin's disease. Billings and Rosenow have also reported the cultivation of a diphtheroid organism from a case diagnosed histologically as lymphosarcoma. In the chloroma case there was noted at operation, also, the same marked interglandular sclerosis seen in Hodgkin's disease. These findings practically force the suggestion, open to further proof or disproof, that these diseases differ but quantitatively and not qualitatively.

To summarize our picture and revise our theory, Hodgkin's disease is an infectious disease due to a diphtheroid organism, the *Bacterium Hodgkini.* There may often be found a primary lesion at the portal of entry. While in some cases the organisms may remain for a long time localized in the vicinity of the portal of entry, in other cases they early gain entrance into the general circulation, and may be widely distributed. The organism and its toxin show a special affinity for lymphoid tissue, and produce in this the characteristic changes of Hodgkin's disease, changes varying somewhat according to the intensity of the toxin, but resulting ultimately in the sclerosis of the glands. There is at the same time an interglandular inflammatory process, at times very acute, but resulting finally in a dense sclerotic tissue. There are also characteristic blood changes in the disease.

The glandular changes can then be considered only as the result of a toxic action, and contribute to the patient's death merely incidentally, when certain gland groups are extensively enlarged. The cells of the enlarged glands, though atypical, show none of the antagonism to the other body cells characteristic of malignant neoplasms.

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THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

IX. THE CHANGES IN THE BONE MARROW AFTER SPLENECTOMY.*

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In connection with the various investigations[1] carried out in this laboratory on the subject of the spleen in its relation to blood destruction and regeneration, the bone marrow of splenectomized dogs has been examined with a view to determining the compensatory or other changes following the removal of the spleen. As the material now consists of marrows representing periods varying from three weeks to twenty months after splenectomy, we consider our study sufficiently comprehensive to justify a detailed report.

In the literature of the subject, the references to changes in the bone marrow following splenectomy are for the most part casual and presented but incidentally in connection with the associated changes in the lymph and hemolymph glands. In Warthin's (1) collection of the literature up to 1903 the following references occur: Tizzoni and Fileti (2) (1880) and Tizzoni (3) (1882) observed in splenectomized dogs a transformation of the fatty marrow of long bones into red marrow. Mosler (4) (1882), working likewise with dogs, concluded that following splenectomy there may be compensatory action on the part of both lymph glands and bone marrow, the latter appearing to play an important part. In one animal ten months after splenectomy the bone marrow resembled that of leukemia. This change, however, was not constant. Laudenbach (5) (1893) observed in one dog (ten to twelve years of age), with severe anemia, signs of increased blood formation in the marrow 145 days after splenectomy. Ceresole (6) (1895), on the other hand, found in splenectomized rabbits no clearly defined new formation of the marrow. Warthin (1) (1903) states that after splenectomy in the sheep and goat slight lymphoid changes in the fatty marrow occur, but he gives no histological description. Of these changes he says:

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[1] *Jour. Exper. Med.*, 1912, xvi, 363, 376, 758, 769, 780; 1913, xviii, 487, 494, 665.

"The beginning lymphoid changes in the fatty bone marrow in the second and fifth month after splenectomy, are to be regarded as compensatory only for the increased destruction of red blood cells and not for any abrogated splenic function of red cell formation."

Other references may be found to changes in the bone marrow in the presence of diseases of the spleen in man (7) and in experimental anemias of animals with or without splenectomy, but few findings after simple removal of the normal spleen are available. Among the latter are Pugliese's (8) observation that after total splenectomy the bone marrow of the hedgehog becomes filled with giant cells. This change Foa (9) has found not to be characteristic of the rabbit. Vulpius (10) who in 1894 reviewed thoroughly the subject of the surgery and physiology of the spleen, and adds some experimental observations, supports the theory of increased activity of the bone marrow after splenectomy. Winogradow (11) found red marrow in the long bones of a dog 132 days after splenectomy, but yellow marrow was present in two after 517 and 760 days respectively, though one of the latter was slightly streaked with red. Hodenpyl (12) in the description of a case of absence of the spleen in man makes no mention of the bone marrow. Taylor (13) describes the marrow of two splenectomized dogs; that from an animal receiving albumoses by mouth and by hypodermic injection, and killed after nine months, was red; a second splenectomized animal, not receiving albumoses, showed a yellow marrow at the end of one year. Freiberg (14) states that he found red marrow in splenectomized animals, and Gibson (15) notes that in a dog killed five and a half months after being deprived of the spleen, the marrow was apparently in the process of change from yellow to red.

In some of these accounts brief mention is made of the increase of giant cells or of pigmented cells or of the numerical relations between the myelocytes and the white and red cells, but we have been unable to find an adequate account of the histology of the bone marrow after splenectomy based on modern conceptions of the cytology of this tissue. Histological descriptions exist, but they are either brief and fragmentary or are based on views current before the attainment of our present detailed knowledge of the morphology of the cells of the blood.

METHODS.

Our studies are based chiefly on the changes in the marrow of the long bones and particularly in that of the femur. As this marrow in the adult dog is normally fatty, objection may be raised against its use, and to overcome this objection, we attempted to study the marrow of the compact bones. The methods of decalcifying the tissues have, however, in our hands, failed to yield satisfactory histological preparations. The alternative, the use of film preparations obtained successively at intervals over long periods of observation being impracticable, the study of cover-glass preparations was limited to a single observation at the time of the death of the animal. At the same time, however, in many instances marrow squeezed from the ribs has been obtained in sufficient amount to section and thus to allow a comparison with changes in the fatty marrow. We have, however, depended largely upon the study of sections of the marrow of the long bones and in particular of the femur. We are satisfied, as the result of our study of the marrow from a large number of normal dogs, that this is, after all, the most rational method of studying compensatory changes, for it is unusual even in a definitely fatty marrow not to find numerous centers of blood-forming cells. These may be limited to the periphery of the marrow or be scattered throughout, but whatever their position they afford an excellent starting-point for the study of increased cellular content as well as of changes in the character of the cells. The fatty marrow is of especial value in the study of the late changes, for in well fixed and well stained marrow there can be no doubt about the change from a purely fatty marrow to a red marrow rich in cells. This is so striking as to remove all doubt which exists when one examines the marrow of compact bone, as of the ribs or vertebræ, by either the section or cover-glass method.

We have worked exclusively with the marrow of the middle third of the femur, avoiding the marrow at either end, partly on account of its bony nature, but chiefly because of the occasional occurrence of more or less red marrow at the ends of the shaft. As only adult dogs have been used, we feel that the constant use of the middle portion of the marrow allows fairly comparable results. In removing the marrow half the circumference of the bone through the greater part of its length has been chipped away, and after separating the marrow from the bone and cutting it at either end it has been easily removed as a solid cylinder by gently rolling it on to a piece of filter paper. In carrying these tissues through the process of fixation and imbedding, the filter paper, which is firmly adherent to the marrow through the coagulation of the attached blood, allows the necessary manipulations without injury to the marrow itself. The routine procedure has been to fix in Zenker's fluid without previous decalcification, imbed in paraffin, and stain with eosin and polychrome methylene blue. Other stains have, however, been used when necessary to bring out certain details.

RESULTS.

It may be stated at the outset that we have found no evidence of an early change in the bone marrow. Splenectomy does not cause, as do successive hemorrhages and hemolytic poisons, a rapid change of fatty marrow to red marrow. This latter change we have produced readily and rapidly in non-splenectomized control animals by the use of specific hemolytic serum and by causing hemorrhage, but we have never seen a frank change from yellow to completely red marrow in the ordinary course of events in the splenectomized animal until many months, usually six or more, had elapsed, and this despite the fact that many of the animals have had, as has been shown in our earlier work, a moderately severe anemia. This anemia has frequently been of as severe degree as that caused by severe successive hemorrhages in the normal dog, but changes in the marrow analogous to those caused by hemorrhage have not been evident in the earlier periods following splenectomy.

In this connection it may be recalled that the anemia of splenectomy in the dog follows a gradual downward course for three to six weeks, the decrease in hemoglobin being relatively more marked than the decrease in red cells, and that an equally gradual repair causes the red cell count and hemoglobin content to approach normal after three to four months or more. At the same time there is a transient initial leucocytosis due chiefly to polymorphonuclear leucocytes, and a more or less constant lymphocytosis with a late eosinophilia. Not infrequently the eosinophils disappear from the circulating blood from the third week until the end of the third month.

We have, therefore, in the course of our studies attempted to determine whether the hyperplasia in the bone marrow after splenectomy is compensatory in the sense of (1) overactivity in red cell formation chiefly, or (2) peculiarly active in the formation of the white cells of the blood, or (3) in the sense of an orderly reproduction of a new marrow with normal activities in the formation of all cells arising within it.

THE NORMAL MARROW OF THE FEMUR OF THE DOG.

In our study of the marrow of both normal and splenectomized animals we have used as a basis for orientation Bunting's (16) conception of erythrogenetic and leucogenetic centers, Muir's (17) descriptions of erythroblastic and leucoblastic reactions, and have received also much aid from Dickson's (18) study of the cytology of marrow. The arrangement described by Bunting is by no means a constant and definite one, but in the masses of marrow cells may be seen groups composed mainly of myeloblasts and surrounded at times by a nearer zone of myelocytes and an outer zone of leucocytes; in other groups with the same center, the outer zone may be made up of nucleated red cells with a still more distant zone of normocytes. We are not convinced that centers for the production exclusively of red cells or of white cells exist, for frequently an intermingling of the two types is seen in one center, but this conception of definite centers is of great assistance in the interpretation of marrow changes.

The study of the marrow of the femur from many normal dogs has led to our recognition of four definite groups of cells: (1) Groups of undifferentiated cells and myelocytes. These lie between fat cells and seem to be in no way connected with blood channels. In all these centers the cells of the connective tissue reticulum are in evidence. (2) Groups of the character described above, but with a peripheral accumulation of cells in which those of the leucocyte series predominate. (3) Groups as in (1), but with a mantle of cells in which those of the erythrocytic series are most in evidence. (4) Groups as in (1), but with an indiscriminate mingling of cells of red and white series.

These groups cannot always be differentiated for not infrequently an indiscriminate mingling of cells obscures the recognition of centers. Moreover, at times may be seen groups composed purely of white cells or of red cells without myelocytic centers. We have, however, found that search for the groupings described greatly facilitates the study of complex marrow pictures and leads readily to a decision as to whether leucoblastic or erythroblastic activity predominates.

In one respect the study of normal marrow has not helped us greatly. Megakaryocytes and polykaryocytes are so infrequent in the normal fatty marrow that we have no basis, in regard to them, for a comparison with hyperplastic marrow. The same holds true for the large endothelial cells which are phagocytic for red cells and are found so frequently in hyperplastic marrow to contain remnants of red cells and fragments of pigment.

THE MARROW OF SPLENECTOMIZED ANIMALS.

In table I the general results of our observations are presented. The terms "yellow" and "red" refer to the gross appearance, not of the surface of the marrow, but of the cross section. "Slight streaking" and "streaked" refer to an intermingling of yellow and red marrow. A marrow is described as "red" only when it is uniformly so. As will be seen by a comparison of gross and microscopic appearances, a marrow "yellow" to the naked eye may, microscopically, show evidence of beginning hyperplasia. The early changes are indicated by the word "slight." The word "complete" indicates that only an occasional fat cell is seen microscopically. "Almost complete" means that fat cells are present in the proportion of one part to nine of marrow cells, in the surface area of sections studied. Several purely fatty marrows represent-

TABLE I.

Hyperplasia of the Marrow of the Femur after Splenectomy.

Dog No.	Period after splenectomy.	Gross appearance.	Microscopic change.
50	24 dys.	Yellow	Slight.
23	39 dys.	Yellow	None.
21	40 dys.	Yellow	Slight.
86	42 dys.	Yellow	Slight.
79	60 dys.	Yellow	Slight.
82	63 dys.	Slight streaking	Slight.
17	84 dys.	Yellow	Slight.
10	6 mos.	Red	Complete.
39	7 mos.	Red	Complete.
32	8 mos.	Yellow	Slight.
44	9½ mos.	Yellow	None.
41	10 mos.	Yellow	None.
24	1 yr.	Red	Complete.
59	1⅓ yrs.	Red	Complete.
57	1½ yrs.	Streaked	Partial.
33	1¾ yrs.	Red	Almost complete.
51	1⅚ yrs.	Yellow	Slight.

ing periods between five and twenty-four days after splenectomy are not included in the table.

The bone marrows representing the earlier periods of splenectomy, in that they show practically no changes, may be dismissed briefly. This is true of a series from animals killed at various intervals from five days to three months. Some of these marrows cannot be distinguished from those of the normal dog. In others, slight replacement of fatty tissue is seen. Thus, one representing the twenty-fourth day shows here and there between the fat cells single rows of blood-forming cells with now and then clumps of ten to thirty or more. These areas are neither purely erythrogenetic or leucogenetic, though in some of the groups with an older type of cells there is a predominance of polynucleated cells. The endothelial cells of the reticulum not infrequently contain large masses of old blood pigment.

Another, representing the fortieth day, presents practically the same appearance with a tendency, however, to greater erythrogenesis. On the other hand, a thirty-nine day dog shows a simple fatty marrow with no evidence of active blood formation. Three other marrows of this period, however, show already the early stages of hyperplasia. In one of these (forty-second day) showing a slight general hyperplasia, both types of cell groups can occasionally be isolated, but usually the groups are mixed. Greater numbers of eosinophil cells, both myelocytic and polymorphonuclear, are present than have been evident in earlier periods. A number of cells throughout the section correspond to Longcope's (19) small lymphocytes and a smaller number to Longcope's large lymphocytes. The small lymphocytes are not, however, in pure groups. The picture as a whole is more one of leucogenesis than of erythrogenesis. Very few giant cells are seen and only occasional phagocytes. Polymorphonuclear leucocytes are abundant.

In another marrow of the sixty-third day, a moderate peripheral hyperplasia of mixed type is present. Marked congestion is evident between the fat cells, and in places near the periphery there is hyperplasia; in some places the erythrocytes appear to be outside the vessel, forming distinct hemorrhages. A few phagocytes are present, but giant cells are rare. Polymorphonuclears are frequent and of mature development. At the periphery erythrogenesis seems to predominate over leucogenesis. Eosinophils and lymphoid cells are not conspicuous.

A marrow of the sixtieth day shows less hyperplasia, but leucocytic reaction is more evident, though erythrogenesis is active. Scattered throughout the section are many small lymphocytes, but nowhere are these seen in solid clumps. Numerous deposits of pigment are seen.

Again on the eighty-fourth day an essentially fatty marrow shows a narrow cellular strip at the periphery in which erythrogenesis is active. Here and there leucogenesis predominates, but in the main the process is erythrogenetic. A few nucleated red cells of the megaloblastic type are found, but the more mature normoblasts are more abundant. In some centers radiating lines of four or five normoblasts are seen. Few giant cells are present.

The changes of the fourth and fifth months after splenectomy are not represented in this study. Well marked hyperplasia is, however, present in bone marrow representing periods of 6, 7, 12, 17, 18, and 20 months after splenectomy. On the other hand, two marrows representing respectively nine and a half and ten months show no departure from the normal fatty marrow, and in a third (eight months) only slight hyperplasia is evident. In the latter are areas composed almost entirely of cells of the myelocyte or premyelocyte type with some evidence of the formation of both red cells and polymorphonuclear leucocytes. The picture suggests a proliferation of the primitive cells of the marrow without, however, a very active function on their part. With evidence of well marked hyperplasia at six and seven months and after a year and a year and a half, it is impossible to explain its failure in these three animals representing the 8th, 9th, and 10th months respectively.

The best opportunity of studying the late changes is presented by material from six animals, representing the period from six to twenty months, in all of which the fatty marrow of the femur was transformed entirely or in large part into red marrow. The histological picture of each of these will be given in detail.

Dog 10.—Splenectomized May 20, 1913. Before operation the red cells numbered 6,910,000 and the hemoglobin was 105. The severest anemia was about

July 21; red cells 4,240,000, hemoglobin 62. On Sept. 11 the figures were 5,220,000 and 92. Later the animal became pregnant and anemia recurred, the picture on Nov. 18 being red cells 4,410,000, hemoglobin 78 per cent. On Nov. 24 the animal was chloroformed. At autopsy the medulla of both femurs presented a deep red marrow.

Histological Examination.—A uniformly cellular tissue is seen with occasionally a fat space here and there at the periphery. For the most part this marrow is as definitely cellular as is, for example, a lymph node or the spleen, and indeed it has the appearance of the pulp of the latter organ in the new born puppy. In this cellular mass, which at first appears to present a hopeless confusion of cells, it is not difficult to resolve the cells into more or less distinct proliferating centers. The arrangement is by no means a definite one, but in the patchwork of cells one sees groups which correspond to Bunting's description. In speaking of these centers we shall refer to them as erythrogenetic or leucogenetic, according to whether red cells or polymorphonuclear leucocytes predominate in the mass of cells surrounding the center in question. We have made no attempt to distinguish in these centers, which may include 6 to 10 or 20 to 30 cells, between the finely granular neutrophil myelocyte and the non-granular basophil cell from which it is supposed to arise. In these centers mitotic figures may occasionally be seen but only after prolonged search. It is also in these centers that old blood pigment, which is quite abundant in this marrow, is deposited; its deposition in the loose vascular tissue elsewhere has not been observed. The erythrocytic centers appear to be more active than the leucogenetic. This impression is based on the fact that about a mass of myelocytes composed of twelve to fifteen cells may be seen twenty-five to thirty nucleated red cells and a small number of normocytes, while about the leucogenetic centers comparatively few leucocytes are seen. The red cells in question vary in size and show changes from the megaloblast to the normocyte. It is not to be supposed that about erythrocytic centers no leucocytes occur. A few are always present; for example, among the twenty-five to thirty cells mentioned above, eight polymorphonuclear leucocytes could be clearly distinguished. Sometimes on one side of a center nucleated erythrocytes may be grouped, and on the other leucocytes with little intermingling. This suggests simultaneous formation of the two cells in one cell center. When this occurs the number of red cells is always greater than the number of leucocytes, in the proportion of 30 to 8. All through the section are lymphoid cells, usually single and of the small variety. Giant cells are frequent and a few show inclusions of polymorphonuclear leucocytes. Cells containing such inclusions have a broad homogeneous gray staining protoplasm suggesting necrosis. There is considerable pigment, but not many phagocytic endothelial cells are seen. Normoblasts are seen free in the capillaries. Smears from the marrow of the ribs show active erythrogenesis, and, on the whole, much the same cellular picture as the marrow just described. In the rib marrow a considerable number of eosinophils, chiefly polymorphonuclears, are also seen. In connection with the activity in the formation of red cells shown by the marrow, it is significant that the blood count six days before death was 4,100,000, and the hemoglobin 78 per cent. On Sept. 11, four months after splenectomy, the figures were 5,240,000 and 92. In other words, despite the hyperplasia of the bone marrow the animal exhibited a late anemia, two and a half months after recovery from the initial anemia, following splenectomy. This may have been due to the drain occasioned by the intervening pregnancy,—an unfortunate complication from the point of view of the study of the blood. The fact remains, however, that the marrow is actively forming normal red cells. The anemia was, therefore, not due to insufficient erythrogenesis in the marrow.

Dog 39.—On Apr. 7, 1913, before splenectomy, the red cells numbered 6,528,000, and hemoglobin was 110 per cent. The lowest point of anemia was reached on June 3, the red cells numbering at that time 3,650,000; the hemoglobin was 62 per cent. By July 7 the blood picture had improved (red cells 5,080,000, hemoglobin 88 per cent.), but on Sept. 11, a late recrudescence of the anemia gave red cells 4,040,000 and hemoglobin 68 per cent. The animal was killed on Nov. 15. The bone marrow of the femur was of a definite red color. The anemia did not affect the general nutrition of the animal. On Apr. 7 the weight was 12,800 gm.; on Nov. 15 it was 13,950, and the adipose tissue was abundant.

This animal, representing practically the same period after splenectomy and the same changes in the blood, presents very much the same picture in the marrow. Of minor importance is the fact that the marrow is not so cellular, the proportion of cells to fat being in the ratio of about 3 to 2; also the myelocytic centers are not so pronounced, but in other respects the marrow is the same. Many giant cells are present but lymphoid cells are rare. The formation of red cells and leucocytes is perhaps not so rapid; that is, the numbers about any one center are not so great but, on the other hand, the activity of the marrow in connection with the former is sufficient to exclude the possibility of the bone marrow being responsible for the later development of anemia.

Dog 24.—This animal was splenectomized on Feb. 10, 1912, and was used for the injection of hemolytic immune serum on Mar. 20 and again on Apr. 7. On June 28 it had recovered from the anemia then produced (red cells 5,650,000, hemoglobin 89 per cent.), and on July 15 it was treated with sodium oleate. On Sept. 26 the red cell count was 5,780,000, and hemoglobin 90 per cent. On Feb. 19 the blood picture had improved (red cells 6,048,000, hemoglobin 110 per cent.), and at this time hemolytic serum was again injected. The animal was chloroformed on Mar. 4, 1913. The lapse of time since splenectomy was, therefore, thirteen months. At autopsy a red marrow was found.

Owing to the use of various hemolytic poisons, the bone marrow of this animal may have been influenced by other factors than the absence of the spleen. The histological picture, however, is so in accord with the marrow of simple splenectomy that, with this explanation, it is included in the series.

Histological Examination.—The marrow is uniformly solid with no fat spaces visible in any of the sections examined. It does not, however, appear to be as cellular as the marrow of dogs 10 and 39. This difference is caused by a greater congestion and distention of the blood vessels, a slight increase in the reticulum, and a lessened tendency of the myelocytic tissue to be grouped in large centers. Erythroblastic centers are very prominent and very active; leucogenetic centers, on the other hand, are made out with difficulty. Lymphoid elements are rare. Many cells of the myelocytic type are seen with coarse basic granules and short threads in the nucleus, and with little or no protoplasm. In close relation to these are sometimes seen degenerated mitoses, but whether all these masses can be so interpreted is not clear. These degenerative changes are doubtless the result of the last injection of hemolytic serum.

Dog 59.—On July 24, 1913, this animal was splenectomized and used for the study of the progressive anemia following this procedure. On Dec. 7, 1912, the highest point of recovery was reached (red cells 5,250,000, hemoglobin 105 per cent.). Continued observation showed a slight decline to 5,200,000 red cells and 86 per cent. of hemoglobin on May 21, 1913, on which date the animal was used in an experiment with sodium oleate. From the moderate anemia caused at this time, the animal quickly recovered, the blood examination on June 9 showing 5,050,000 red cells and 86 per cent. hemoglobin, the condition slightly improving as to hemoglobin content until Nov. 18, 1913, when red cells were 5,100,000 and hemoglobin 101 per cent. The animal was chloroformed on Nov. 24. At autopsy the bone marrow of the femur was soft, succulent, and dark red in color. In connection with the general condition of this animal it is of interest to note that in the last seven months its weight increased from 10,450 to 12,580 gm., and that adipose tissue was very abundant. The administration of sodium oleate introduces a possible disturbing factor, but as this was given six months before death, it is not considered, in view of our other results, a serious matter.

Histological Examination.—The marrow shows some fat cells, the proportion of marrow cells to fat being about 10:1. Nothing new is presented. Leucogenesis and erythrogenesis proceed at about equal rate, the latter being a little more active. Mitotic figures are seen not infrequently, but the type of cell in which they occur is not always evident. Myeloblasts seem to be more abundant than usual. Giant cells are fairly abundant, but lymphoid cells are rare.

Dog 57.—On June 23, 1912, the blood of this dog contained 5,350,000 red cells per cubic millimeter, and 98 per cent. hemoglobin. On July 2 the spleen was removed. The resulting anemia reached its lowest point (red cells 2,970,000, hemoglobin 50 per cent.) on Aug. 5. On Oct. 24, two days after the blood count showed 5,240,000 red cells and 90 per cent. hemoglobin, the animal received sodium oleate intravenously; a very slight anemia (fall in hemoglobin to 62 per cent., but no change in red cells) resulted. In Jan., 1913, the red cells were 5,206,000, hemoglobin 110 per cent., and with slight variations this higher level was maintained, accompanied by an increase in body-weight, until Dec. 12, 1913, when the animal was chloroformed. At autopsy the animal was found to have a large amount of adipose tissue; the bone marrow of the femur was definitely reddish in color with faint yellowish streaks. As the sodium oleate given four months after splenectomy and fourteen months before death produced only a slight transient change, we consider that the bone marrow represents the effect of splenectomy only.

Histological Examination.—The relation of the fat to cells is about 1 : 1; otherwise nothing new is seen. The marrow is very active, leucogenesis and erythrogenesis being equally prominent. Phagocytic cells and masses of old blood pigment are quite numerous, as are also giant cells. More abundant than in other marrows are eosinophils of the myelocytic type. Lymphoid cells are not conspicuous.

Dog 33.—This animal was splenectomized on May 14, 1912, the blood examination on the previous day showing 4,950,000 red cells and 85 per cent. hemoglobin. The anemia following splenectomy reached its lowest point on June 28 (red cells 3,550,000, hemoglobin 52 per cent.). On Sept. 20 the red cells had risen to 5,490,000 and hemoglobin to 95 per cent. In Nov., 1913, the animal passed successfully through pregnancy. In Jan., 1914, as the animal had developed mange, it was chloroformed. The blood examination on the preceding day was red cells 4,480,000, hemoglobin 70 per cent. At autopsy the bone marrow of the femur was deep red in color. It should be stated that one and two months before splenectomy the animal had received injections of hemolytic serum. From our studies of the effect of hemolytic serum in the normal dog, we do not believe that these injections, nearly two years before death, are in any way responsible for the hyperplasia of the marrow.

Histological Examination.—This marrow differs in no way from those of dogs 57 and 59 described above.

Dog 51.—The spleen was removed on May 31, 1912, and on June 26 hemolytic serum was administered. From the anemia thus produced the animal made a slow recovery, but after 200 days the blood examination showed 6,200,000 red cells and 110 per cent. hemoglobin, as compared with 6,210,000 red cells and 100 per cent. hemoglobin before splenectomy. On Mar. 26, 1914, when the animal was chloroformed, its weight was 9,750 gm. as compared with 8,270 gm. at the time of splenectomy, and 8,120 when hemolytic serum was administered. The notes made at the autopsy refer to the large amount of adipose tissue, the normal appearance of the lymph nodes, the absence of supernumerary spleens, and the presence in the long bones of a distinctly yellow fatty marrow.

Histological Examination.—The marrow shows a very slight hyperplasia with large numbers of leucocytes and deposits of blood pigment.

DISCUSSION.

In view of the slight changes seen in the bone marrow during the early periods after splenectomy, it appears that neither during the period of anemia and consequent repair nor in the period of hyperplasia of bone marrow are nucleated or other irregular forms of red cells found frequently in the peripheral blood. Careful differential counts of three dogs at regularly spaced intervals for 138 days failed to reveal in two the presence of nucleated red cells, and in one they were found only five times, the largest number seen in one count being three. In a large number of other animals in which differential counts were made at irregular intervals, changes in the red cells have been found very rarely; in one dog five weeks after splenectomy five normoblasts and two megaloblasts were found (in 100 cells) with evidence of poikilocytosis and polychromatophilia, and a week after one normoblast and one megaloblast. These findings correspond to the first days of beginning repair, the red cells and hemoglobin having a few days before reached the lowest level observed during the experiment; hemoglobin 50 per cent., red cells 2,970,000. In another, two months after splenectomy, again at the stage of beginning repair (hemoglobin 92, red blood cells 3,650,000) five nucleated red cells were found, and a polychromatophilia was evident. In no instance did these findings persist for any length of time. If they have any significance it is that they indicate the period of beginning repair.

It is difficult to bring the changes in the bone marrow into relation with the changes in the peripheral blood. If the hyperplasia of the bone marrow is compensatory to increased blood destruction, or decreased blood formation, one would expect definite hyperplasia to be present in the earlier period, during the first three months after splenectomy, at a time when the anemia is evident and repair is taking place, and not after six months to a year or a year and a half when the blood picture is normal. It is true that in two of the animals (dogs 10 and 39) a late recrudescence of anemia occurred and the marrows of these animals were obtained during this period, but this was not the case in other animals of the series and is not characteristic of the late periods after splenectomy. It is therefore impossible, on account of the late development of hyperplasia in the marrow, to explain its occurrence as compensatory to the anemia following splenectomy.

Likewise we cannot accept Warthin's theory based upon his study of sheep and goats. In these animals Warthin found hyperplasia of the marrow to occur several months after splenectomy and to be associated with evidence of increased destruction of red blood cells in the lymph and hemolymph nodes. This destruction, greater than that in the primitive spleen, is responsible, he believes, for the anemia following splenectomy, and this is in turn compensated by increased activity in the bone marrow. We have found little to support this theory in our studies of the dog. The lymph nodes, as well as the endothelial cells of the liver, as we have shown elsewhere (20), are indeed more active after splenectomy than in the normal animal in the phagocytosis and destruction of red cells, and this is very evident when large numbers of red cells are injured, as by the administration of a hemolytic poison; but in the ordinary course of events, after splenectomy, the lymph nodes present no evidence of excessive blood destruction. An occasional cell containing one or two red cells may be seen and small amounts of old blood pigment are occasionally demonstrable, but of excessive hemolysis there is no evidence. Microchemical tests for iron in the lymph nodes of fifteen splenectomized dogs showed a considerable amount of iron in three, slight amounts in five, and none in six. The animals examined represented periods of eleven days to twenty-two months after splenectomy. In the lymph nodes of eight normal animals similarly examined, moderate amounts of iron were found once, slight amounts three times, and in four, none. It is evident, therefore, that in the dog the iron content of the lymph nodes after splenectomy differs little from normal. The liver likewise shows no increased deposition of iron. Of fourteen livers from splenectomized dogs, four showed slight depositions of iron in the Kupffer cells, while ten showed none. At the same time the livers of six normal dogs were similarly examined; in three slight deposits of iron were found, and in three none. For this reason, and because the anemia is not persistent and progressive we cannot support the theory that the hyperplasia of the marrow is compensatory to abnormal blood destruction in the lymph nodes.

Another possible explanation is that the bone marrow, in the absence of the spleen, is concerned in the storing and utilization of iron. There is no doubt that, in the intact animal, iron set free in the dissolution of red cells is stored in the spleen. After splenectomy a readjustment in the storage of iron takes place, and there is some evidence that for a short time after the removal of the spleen iron is lost to the body. It is possibly this disturbance of iron utilization that is responsible for the early transient anemia. Our investigations[2] show, however, that this disturbance of iron utilization is transient and that after a few weeks the elimination of iron in the splenectomized animal differs in no way from the process in the normal animal. This suggests naturally that the storage of iron in the absence of the spleen is taken over by other tissues. As microchemical tests for iron showed no definite increase of iron in the lymph nodes and liver it seemed probable that the bone marrow might be the chief depot of iron storage. Such a view was supported by the fact that all hyperplastic bone marrows contain large amounts of altered blood pigment, sometimes free, but occurring, for the most part, in large phagocytic cells. The activity of these phagocytic cells presumably in transforming the iron of old blood pigment in order that it may be reutilized by the red cells, might, it was plausible to suppose, stimulate the other functions of the bone marrow, that is, the erythrogenetic and leucogenetic functions and cause eventually a replacement of the fatty marrow by a very cellular red marrow.

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[2] To be presented in detail in a future communication.

In order to prove this hypothesis it was necessary to obtain some idea of the iron content of these marrows. Direct chemical analysis was out of the question on account of the small amount of material available and the variations in blood and bone content of different marrows. We therefore made a comparative study based on the use of the microchemical reaction for iron. This demonstrated at once that all red marrows in our series have a large content of iron, and that fatty marrows contain very little or no iron. On the other hand, when the marrows of non-splenectomized dogs rendered hyperplastic by anemia or infection were examined it was found that these also had a large iron content. Thus in a group of 17 non-splenectomized dogs iron was present in the marrow in large amounts in 4, in moderate amounts in 2, in small amounts in 4, and in 7 none was found. On the other hand, in 27 splenectomized dogs, iron was present in large amounts in 10, in moderate amounts in 3, in small amounts in 4, and absent in 10.

In both groups the amount of iron was in direct proportion to the degree of hyperplasia. These observations point therefore to the conclusion that a red marrow is always rich in iron, but it is impossible to say whether the cellular hyperplasia or the iron deposition is primary. Under the circumstances, it is also impossible to conclude that the late hyperplasia of marrow following splenectomy is an attempt to conserve iron. Moreover, the irregularity of our results as shown by the failure of hyperplasia in four animals, representing respectively the 8th, 9th, 10th, and 22d months after splenectomy, prevents in the present state of our knowledge an adequate explanation of the cause of the transformation from yellow to red marrow.

The divergent results in this study are characteristic of all phases of experimental work on the spleen and doubtless are to be explained by the fact that removing the spleen takes away only one organ of a system composed of liver, spleen, lymph nodes, and bone marrow, and that the interrelations which exist in this system may or may not under varying circumstances bring into play compensations of the greatest importance in determining the degree of blood destruction or regeneration and therefore the degree of change in the bone marrow.

A search of the literature of splenectomy in man, although it reveals evidence of the occurrence of red marrow in various forms of splenic anemia (7), offers little of importance concerning the changes which occur in the bone marrow after removal of the normal spleen, Several references (21) are made to the occurrence of pain in the long bones after splenectomy, and by some this has been assumed to be evidence of hyperplasia within the rigid bony canal. The only note of the direct examination of the bone marrow after splenectomy is that of Riegner (22), who found active proliferation of the marrow of the femur in a man whose leg was amputated for gangrene four weeks after splenectomy for trauma. It is therefore impossible, on account of this paucity of data concerning the changes in man, to bring them into relation with our experimental results.

CONCLUSIONS.

Splenectomy in the dog causes, as a rule, a transformation of the fatty marrow of the long bones to a richly cellular red marrow.

During the early periods, one to three months, the change in the marrow is slight and either focal or peripheral; after six to twenty months the replacement of fat by marrow cells is complete or nearly so. Exceptions were, however, seen in four animals representing the 8th, 9th, 10th, and 22d months, respectively. The evidence at hand does not support the theory that this hyperplasia is compensatory either to the anemia caused by splenectomy or to an increased hemolysis in the lymph nodes. It is possible that it may be a concomitant of the activity of the bone marrow in taking over, in the absence of the spleen, the function of storing and elaborating the iron of old blood pigment for future utilization by new red cells, but our studies do not fully support this view.

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THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

X. CONCERNING THE SUPPOSED REGULATORY INFLUENCE OF THE SPLEEN IN THE FORMATION AND DESTRUCTION OF ERYTHROCYTES.

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In the older literature of the physiology of the spleen appear numerous references to differences in the composition of the blood of the splenic artery as contrasted with that of the splenic vein, and in the recent literature observations are made upon the occurrence of a specific hemolysin in extracts of splenic pulp. The possible bearing of these observations upon the various studies[1] of the spleen reported from this laboratory during the past two years led to a critical examination of the general literature and to certain experiments already described, in the hope that light might be thrown upon some of the difficult aspects of the general problem. A careful study showed that the observations in question are subject to a large experimental error which renders the finding of slight differences in the composition of arterial and venous blood of doubtful value,—a reason why the work has not heretofore been utilized in our reports. Indeed the work probably would have been entirely disregarded if Banti had not recently claimed, in support of his theory of the splenic origin of icterus, that the blood of the splenic vein normally contains more free hemoglobin than the blood of the general circulation. In order to test this and other points we have repeated experiments upon which the assumption that the spleen has a direct or indirect influence upon the red cells is based.

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[1] *Jour. Exper. Med.*, 1912, xvi, 363, 375, 758, 769, 780; 1913, xviii, 487, 494, 665; 1914, xx, 19.

Much of the early work on this subject is not only contradictory but was done before the development of the present exact methods of blood examination. Thus Virchow (1) found fewer red cells in the blood of the splenic vein than in that of the artery; while Malassez and Picard (2) and Emelianow (3) report the opposite. On the other hand, later investigators, Vulpius (4) and Paton, Gülland, and Fowler (5), have found no constant or noteworthy differences.

Considering the spleen as a possible leucoblastic organ, numerous early observers (6) found relatively more leucocytes, especially so called young forms, in the blood emerging from the spleen, than in that entering it. Tarchanoff and Swaen (7) and also Virchow (1) could not find any noteworthy difference, whereas Paton, Gulland, and Fowler (5) noted a constant diminution in the number of leucocytes in the splenic vein as compared with the general circulation. In this connection Bulgak (8), who describes an increase in leucocytes in the splenic vein, states that this is true of the venous blood of all parenchymatous organs. Freyer (9) concludes from his comparative counts that the spleen has nothing to do with blood formation.

The preceding studies refer of course to mature animals. It is accepted that in fetal life the spleen has the power of extensive blood formation, and several reports are at hand to show that the adult spleen may undergo, in the presence of injury to the bone marrow, a myeloid metaplasia (10); that is, that it can regain under pathological conditions its fetal function. Whether or not the spleen may exert this power of blood formation in the adult under normal conditions is doubtful, though still an open question.

Although the spleen certainly destroys red blood cells, as is evident from the presence in it of large cells, phagocytic for erythrocytes, which are increased under certain pathological circumstances, there still exists doubt whether the destruction by phagocytosis is the only method of red cell disintegration. It is stated also that the erythrocytes, in their passage through the spleen, are so acted upon by some unknown substance as to become more susceptible to hemolysis. This is the basis of Bottazzi's (11) hemocatatonistic theory, which has recently received support from Banti (12) and his colleague Furno (13). In the course of an investigation of hemolytic splenomegaly, they studied normal animals and those receiving hemolytic serum and came to the conclusion that free hemoglobin can be demonstrated in the blood of the splenic vein in normal animals as well as in animals receiving hemolytic serum. It was found at times in the blood of other vessels but in less amounts than in the splenic vein. They consider the findings as evidence of hemolysis in the spleen. The red blood cells of the splenic vein were found also to be less resistant to hypotonic salt solution than were those of the general circulation. Observations by Chalier and Charlet (14) on the resistance of red cells in the splenic artery and vein gave different results. Although the venous blood in general was slightly less resistant than arterial blood, this was reversed in the splenic system, so that the blood of the splenic vein was more resistant than that of the splenic artery and much more than the blood of other veins. Hammarsten is also said by Gabbi (15) to have found that the splenic vein blood was more resistant than the arterial. In the observations of Banti and Furno, the reference is to free hemoglobin in the serum and not to the increased hemoglobin content of venous or splenic blood described by several investigators (16).

The claim of Banti and Furno is surprising in that they state that the dissociated hemoglobin of the serum (*"emoglobin disciolta dal siero"*) is not only always present in the splenic vein of normal animals, but in sufficient quantities sometimes to be measured by a Sahli hemoglobinometer. It is to these observations that we have given especial attention in our work.

COMPARISON OF THE ARTERIAL AND VENOUS BLOOD.

Method.—From dogs under ether anesthesia, blood was obtained directly from the splenic artery and the splenic vein. Great care was exercised to disturb the vessels and the organ as little as possible, as it has been shown by Grigorescu (6) and Pribram (17) that the cell content of the blood may be greatly increased by congestion of the spleen. From a nick in the vessel wall of one of the branches of the artery or vein, fresh blood was drawn directly into Thoma blood-counting pipettes and the capillary tube of a von Fleischl hemoglobinometer. From another branch, blood was withdrawn by a syringe and immediately distributed to tubes containing different strengths of hypotonic salt solution designed to test the resistance of the red cells. Some of the blood was also set aside for similar tests with washed cells. For the determination of the presence of free hemoglobin in the serum, blood was collected in three ways: (1) in a paraffined centrifuge tube, (2) in a tube containing potassium oxalate, and (3) by drawing it directly into tubes through capillary points which were then sealed. All three samples were then centrifuged and the serum was examined for hemoglobin by visual inspection and the spectroscope. Smears for differential counts were made at times from the blood flowing directly from the vessel and at times from a drop from the syringe. Finally, tests for reticulated or skeined (young) red blood cells were made. This was done by letting a few drops of blood fall into a solution of brilliant cresyl blue, and, after standing fifteen or twenty minutes, the skeined forms in proportion to the unskeined or mature forms were counted in fresh smears. For the purpose of controls, blood from the femoral vein, and from the capillary circulation by puncture of the skin was occasionally collected.

Results.—The figures for the red and white cells, differential counts, and total hemoglobin in a series of five dogs show that as far as these estimations are concerned the blood of the splenic vein does not differ greatly from that of the artery. The variations are not uniformly on one side and are all within the limit of error inherent in the methods of blood examination.

It is of interest that in these and other dogs, the red cells of the vein, in six of eight animals, showed more or less marked anisocytosis and inequality of staining, which were not seen to the same degree in the blood of the artery. Polychromatophilia was about equal in artery and vein. In two of the eight animals a few normoblasts were found in the splenic vein blood only. Control smears from the femoral vein of four dogs showed changes in the red cells about equal to that of the splenic vein, indicating that these changes are characteristic of venous blood in general rather than a specific change caused by passage through the spleen.

In regard to the presence of free hemoglobin in the serum, if we had depended on one tube only, we should have occasionally found apparent hemoglobinemia, both of the general circulation and of the splenic vein; but as in every set of three tubes, in a series of seven dogs, at least one was free of hemoglobin, we cannot support the view that free hemoglobin in demonstrable amounts is present normally either in the splenic vein or in the general circulation of the dog. Our experience forces us to the conclusion that the findings of other investigators are due to hemolysis after collection or are dependent upon the method of separating the serum.

As regards the resistance of the red cells, of which comparative tests were made on eight dogs, in five no difference was found between artery and vein; in the other three, the venous corpuscles were slightly less resistant. Two control tests with cells from the femoral vein showed these to have the same resistance as those of the splenic-vein.

In seven comparative tests for skeined or reticulated red corpuscles, these were found five times to be more abundant in the splenic vein and twice more numerous in the artery; the differences were never very striking. Five controls from the femoral vein corresponded more closely to the splenic artery counts than those of the splenic vein.

Conclusions.—As a result of the various observations we conclude that the slight differences between the arterial and venous blood of the spleen are within the limits of error inherent in the methods of blood examination and are not to be explained by a peculiar action of the spleen. In some instances peculiarities shown by the splenic venous blood are common to the venous blood of the general circulation. Banti and Furno's observation concerning the presence of free hemoglobin in the blood of the splenic vein is not confirmed.

THE HEMOLYTIC POWER OF SPLENIC EXTRACTS.

The histological evidence of the destruction of erythrocytes by phagocytic cells of the spleen has naturally suggested the possibility of the liberation by these cells of a ferment capable of acting extracellularly. If such a free hemolysin is present in the spleen it should be demonstrable in extracts of the spleen, and during the past few years several investigators have therefore tested the influence of such extracts upon red cells. The methods employed, based on the technique of Korschun and Morgenroth (18), are similar, but the results obtained have been contradictory.

Korschun and Morgenroth found in several organs a hemolytic substance of unknown origin, coctostabile and soluble in alcohol, which did not arise from constituents of the blood serum and was in no way peculiar to the spleen. Nolf (19), on the other hand, found that the hemolytic power of splenic extract was distinctly greater than that of the liver, mesenteric lymph nodes, or kidneys, but only slightly more than that of the lung. This hemolytic substance was specific for the species and was destroyed at 100° C. Achard, Foix, and Salin (20), repeating these experiments, showed that the final solution was strongly acid, presumably as the result of bacterial action, and that control tests made with precaution as to asepsis were uniformly negative. Widal, Abrami, and Brulé (21) in similar experiments could get no hemolysis with fresh extracts used on the day they were prepared; sometimes, also, extracts twenty-four to forty-eight hours old were without effect. From these results they conclude that the hemolytic substance is not a true hemolysin, but the product of cell autolysis. Iscovesco and Zacchiri (22) had previously shown that after placing the mixture of pulp and saline solution in the thermostat for fifteen to twenty hours, the filtered extract, on the addition of red cells and after standing two and one half hours in the thermostat, showed 2.5 to 8 per cent. hemolysis, as determined by the Dubosc colorimeter, and they conclude that the hemolytic power of splenic extracts is unimportant. Weill (23) found a weakly hemolytic substance in extract of spleen that was inactivated at 56° C. and reactivated with guinea pig serum. This was more powerful than a lymph node extract prepared in the same way, but much less powerful than the extract obtained from the spleen by long maceration. The latter was not destroyed below 80° C., and its action was hindered by adding fresh serum. Extracts from lymph nodes prepared in the same way showed only slight hemolytic action and those from other organs were negative. Banti (12) and Furno (13) state that fresh extracts of the normal spleen sometimes have no hemolytic action, and sometimes a weak action which is increased on standing twenty-four to forty-eight hours on ice and is not destroyed by heating to 60° or even to 100° C. They consider it a cytohemolysin, normally present in the spleen in small amounts and much increased after the administration of hemolytic agents. Thus we find that Nolf, Weill, Banti, and Furno find splenic extracts to have a hemolytic action greater than that of other organs. Achard, Foix, and Salin, and Widal, Abrami, and Brulé, on the other hand, fail to find any hemolytic action of the fresh extract, and think it is found only after autolysis or bacterial decomposition of the spleen.

Our experiments were made with extracts from the spleens of three dogs. The technique described by Nolf was followed in the main with several additions in the way of control experiments. On washing through the aorta, it was found that the technique which will give a blood-free kidney or liver will not render the spleen bloodless. Various expedients were tried, therefore, to secure a hemoglobin-free extract. It was found that if the spleen, after washing through the aorta, was cut in small pieces and pounded with a pestle against a wire-meshed sieve placed in a mortar, with the aid of frequent washings with salt solution, a blood-free white mass was obtained consisting partly of reticulum and partly of adherent splenic pulp (extract A, table I). As it was possible that the hemolytic substance might not be retained, or in only small amounts, in this fraction, extracts were also made from that part of the spleen that was mashed through the sieve. This residue was of course distinctly blood-tinged, so that it was necessary, in order to remove the blood, to mix it with distilled water, centrifuge, discard the supernatant fluid, and repeat the process until colorless tissues were obtained (extract B, table I). In each case the material thus obtained was mixed with double the amount of salt solution and placed in the refrigerator. Tests were always made with extracts one or two hours old, a small portion being filtered off for this purpose, and in two instances also after eighteen and twenty-four hours. Control tests were made in one experiment with extracts of liver and mesenteric lymph nodes. As it was possible to wash the latter free of blood before removal from the body, an extract was easily obtained by grinding the tissue in sand with mortar

TABLE I.

Character of extract.	Amount of splenic extract in c.c.								Salt solution control.	Distilled water control.
	1.95	1.5	1.0	0.5	0.3	0.2	0.1	0.05		
1. Dog 1. Fresh spleen extract A......	—	V.S.	0	0	0	0	0	0	0	C.
2. Dog 2. Fresh spleen extract A......	—	0	0	0	0	?	0	0	0	C.
3. Same. Extract B........	—	0	0	0	0	0	0	0	0	C.
4. Same. After extraction in ice chest for 24 hrs......	—	V.S.	V.S.	0	0	0	0	0	0	C.
5. Dog 3. Fresh spleen extract A......	V.S.	0	0	0	—	0	0	0	0	C.
6. Spleen extract A after extraction in ice chest for 24 hrs................	—	0	0	0	0	0	0	0	0	C.
7. Fresh spleen extract (boiled)...............	0	0	0	0	—	0	0	0	0	C.
8. Fresh liver extract........	0	0	0	V.S.	—	0	0	0	0	C.
9. Fresh mesenteric lymph node extract...........	0	0	0	0	—	V.S.	0	0	0	C.
10. Mesenteric lymph node extract after extraction in ice chest for 24 hrs......	—	0	0	?	V.S.	M.	V.S.	0	0	C.

0 = no hemolysis; ? = doubtful hemolysis; V.S. = very slight hemolysis; M. = marked hemolysis; C. = complete hemolysis; — = no test.

and pestle and placing it as before in the ice chest with double the amount of salt solution. In two experiments the tests were made on the corpuscles of the animal furnishing the spleen; in one the corpuscles of another dog were used without a difference in result. The preparation of the washed red blood corpuscles, the dilutions, incubation, and so forth, were made according to Nolf's technique. Each tube contained 0.1 of a cubic centimeter of washed dog's corpuscles with varying amounts of splenic extract made up to two cubic centimeters with normal salt solution. Controls were made with normal salt solution and distilled water. The results are presented in table I.

Conclusions.—Fresh extracts of spleen are devoid of definite hemolytic action. Occasional trivial and irregular results, not to be explained, are found, but these occur likewise in the control extracts of liver and mesenteric lymph nodes. Extracts twenty-four hours old, prepared at low temperature, show little or no increase in hemolytic activity. Boiled splenic tissue, extracted in the cold for twenty-four hours, is inert.

THE INFLUENCE OF THE INTRAPERITONEAL INJECTION OF SPLENIC EXTRACT.

The changes in the blood picture following experimental removal of the normal spleen suggest that changes of interest might be produced by a converse procedure; namely, introduction into the body of the products of splenic activity in the form of splenic extract. If temporary anemia follows removal of the spleen, one might expect that some temporary rise in the red cell count might follow the injection of splenic extract.

The literature concerning the spleen contains very few reports on this subject. Danilewsky (24) found a surprising increase in hemoglobin and red blood cells after a single subcutaneous or intraperitoneal injection of extract of spleen. This increase reached its height in from three to seven days and continued as long as the experiment lasted, usually eight days. In dogs with a dietary anemia, splenic extract caused an even greater rise; for example, of 40 per cent. hemoglobin and almost 2,000,000 red cells. Danilewsky assumed that his results were due to a stimulation of the bone marrow. This influence of the splenic extract was not destroyed by heating.

Danilewsky's work, however, is uncontrolled by injection of other organ extracts, and the rise noted extended over a surprisingly long period of time. Silvestri (25) records a single observation in which a dog, presumably dying from anemia, was apparently saved by the injection of splenic extract. In this connection it must also be noted that the clinical literature of this subject contains several reports (26) of the use, with good results, of extracts of spleen and bone marrow in the treatment of anemia.

Method.—We have tested the effect of splenic extract on four dogs, using as controls extracts of other organs similarly prepared and extracts of erythrocytes.

The usual examinations of the blood were made, and also determinations of the resistance of the erythrocytes to hypotonic salt solution and the percentage of skeined cells. As a rule two counts were made before injection, and daily counts after the injection until the blood picture had returned to normal, usually a period of from three to four days. Extracts were prepared from organs removed aseptically from dogs bled to death under ether anesthesia. The finely chopped organ was ground in a sterile mortar to a homogeneous pulp and extracted with double the volume of salt solution for two hours in the ice chest. Ten cubic centimeters of the filtered extract were injected intraperitoneally into dogs of about the same weight. Defibrinated blood diluted 1 to 20 with normal salt solution was used in ten cubic centimeter amounts to control the possibility of the rise in red cell count being due to the influence of some constituent of the red cells. In no case did peritonitis or other infection result. The result of one of these experiments is shown in table II.

TABLE II.

Date (1914).	Hemoglobin.	Red blood cells.
Feb. 6	102	5,250,000
Feb. 7	101	5,650,000
	(10 c.c. splenic extract No. 16 injected.)	
Feb. 8	110	6,500,000
	(15 c.c. of same extract injected.)	
Feb. 9	110	7,040,000
Feb. 10	105	6,800,000
Feb. 11	96	5,330,000
Feb. 12	95	5,290,000
	(15 c.c. splenic extract No. 88 injected.)	
Feb. 13	101	5,700,000
	(10 c.c. of same extract injected.)	
Feb. 14	104	6,880,000
Feb. 15	98	5,860,000
Feb. 16	96	5,120,000
Feb. 19	106	5,540,000

This experiment shows that intraperitoneal injection of splenic extract causes a sharp rise in hemoglobin and red cell count, lasting only one or two days. This rise is repeated on reinjection of either the same or another splenic extract.

In each of three other experiments with splenic extract an increase in the number of red cells was obtained, but this increase was not always as marked as in the experiment presented; it was nevertheless always greater than that caused by the use of control extracts of liver, kidneys, or blood.

The study of the resistance of the red cells may be dismissed with the statement that no noteworthy differences were found after injection of any extract. The skeined cells also showed no constant change. We had hoped that as the latter are supposed to be young forms of erythrocytes, they would be found to be increased after the injection of splenic extract had caused a rise in the red cell count. Only once, however, when the percentage rose from 0.5 to 2 per cent., was this noticed. On the other hand, in two experiments they were not found at all in the blood after injection.

Intraperitoneal injection of splenic extract is usually followed by an increase in the total number of leucocytes, consisting chiefly of the polymorphonuclear forms. A similar rise occurred in one of three injections of liver and kidney, and in one of two of defibrinated blood. Several grades of transitional cells appeared in increased numbers. Eosinophils were present in increased numbers in two of the four dogs receiving splenic extracts, but were also definitely increased in two of the five controls.

Conclusions.—Intraperitoneal injection of saline extracts of fresh spleen constantly causes a sharp increase in red cell count and hemoglobin content. The rise is evanescent, lasting but one or two days, and may be followed by an equally evanescent drop below normal. Similarly prepared extracts from other organs fail to give this rise. No noteworthy change is found in the resistance of the red blood cells to hypotonic salt solutions, or in the number of skeined or reticulated erythrocytes, after the injections of the various organ extracts.

A temporary increase of polymorphonuclear and transitional leucocytes usually follows the use of spleen extract, but may occur also, though less frequently, after the injection of liver and kidney.

The constant increase of red cells in the peripheral circulation after injection of spleen, in view of the tendency to anemia following splenectomy, suggests that the spleen normally may exert a stimulating effect upon the formation of red cells in the bone marrow.

THE INFLUENCE OF FEEDING SPLEEN TO SPLENECTOMIZED DOGS.

This study complements that just described in that spleen in large amounts was fed to splenectomized animals. The object was to determine whether through the influence of a possible internal secretion of the spleen the anemia following splenectomy might be prevented. The procedure is of course analogous to thyroid feeding in insufficiency of the thyroid gland, and has an advantage over the injection of extracts in that it may be continued over long periods of time without the possibility of the complications occasionally occurring after injection. These experiments, it was hoped, would show whether or not the spleen exerts some effect upon the hemopoietic system through peculiar bodies concerned perhaps in an internal secretion. Thus, if the anemia following splenectomy depends upon the absence of a normal stimulus to the hemopoietic system in general, or to some part of it, as the bone marrow, furnished normally by the spleen, the feeding of normal fresh spleen unmodified by heat or chemicals would supply this secretion and there would be no anemia after the removal of the spleen.

Method.—Five dogs were used. Four of these were given a diet consisting of raw hashed beef spleen, lard, and cracker meal in amounts estimated, according to the weight of each animal, to suit its caloric needs. Of these, three were splenectomized and one served as a control. As an added control, a splenectomized dog received a diet in which casein was substituted for beef spleen. The red cells and the hemoglobin were estimated several times before splenectomy and afterwards counted twice a week for three weeks. After this they were counted every week for approximately five weeks. No preliminary counts were made until a dog had been on the special diet for at least a week, and splenectomy was not performed until two weeks later.

Results.—Of the three splenectomized dogs receiving spleen in the diet, one showed a very slight decrease in red cells and hemoglobin, but the other two developed the usual anemia of splenectomy. Thus one with an initial red cell count of 6,200,000 showed on the 12th day 4,710,000 red cells with return on the 54th day to 6,040,000. This animal received daily 150 grams of beef spleen. The other dog receiving daily 275 grams of spleen showed a change in red cell content of about the same degree. In the splenectomized dog not fed spleen, the red cells fell from 5,550,000 to 4,210,000 on the 19th day with return to 5,060,000 on the 54th day. In this dog the hemoglobinemia reached its lowest level (65 per cent.) on the 12th day, and remained at about that point until the 26th day. In neither of the other splenectomized animals receiving spleen did the hemoglobin fall below 75 per cent. The normal dog, receiving 150 grams of spleen daily, showed no change in the blood.

It is evident that in two dogs, despite the feeding of spleen, an anemia was produced that ran a course similar to that which we have previously shown to be the rule in splenectomized dogs (27). In view of these definite results, the absence of marked anemia in the third splenectomized dog must be considered as the result of factors other than the feeding of spleen.

Incidentally it was found that the resistance of the red cells to hypotonic salt solution was increased in all splenectomized dogs, thus confirming the work of Karsner and Pearce (28).

Conclusion.—The feeding of fresh raw spleen to splenectomized dogs has no clearly defined influence in preventing the anemia which usually occurs after splenectomy.

GENERAL SUMMARY.

1. The blood of the splenic artery and vein shows either no differences, or only such slight irregular variations as may be due to the errors inherent in hematologic methods, or are common to arterial and venous blood of the general circulation.

2. The observation of Banti and Furno that free hemoglobin occurs in the blood of the splenic vein is not confirmed.

3. Extracts of the spleen have no definite hemolytic action *in vitro*.

4. Intraperitoneal injection of fresh saline extracts of the spleen causes in the dog a sharp increase in the number of red cells and the hemoglobin content which lasts for one or two days and may recur on a second injection. Extracts of liver, kidney, and erythrocytes similarly prepared do not give this effect. This observation supports Danilewsky's theory that the spleen may exert a stimulating effect upon the formation of red cells in the bone marrow.

5. On the other hand, the feeding of raw beef spleen to splenectomized dogs over long periods of time has no clearly defined influence in preventing the anemia which usually follows splenectomy.

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THE RELATION OF THE SPLEEN TO BLOOD DESTRUCTION AND REGENERATION AND TO HEMOLYTIC JAUNDICE.

XI. THE INFLUENCE OF THE SPLEEN ON IRON METABOLISM.*

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This investigation was undertaken to determine whether the tendency to anemia in splenectomized dogs and the delayed regeneration of the blood after the administration of hemolytic agents to such dogs[1] might be due in part to some influence of the spleen upon the iron metabolism, as has been claimed by Asher (1).

Our present knowledge concerning iron metabolism may be summarized as follows: Iron is absorbed only to a very limited extent from the gastro-intestinal tract, so that when abundant in the food it passes for the most part unchanged from the intestine in the feces. As much as is absorbed is taken up chiefly from the small intestine and carried by the lymph to be deposited in the liver and to a lesser extent in the spleen, bone marrow, and perhaps elsewhere, and this occurs whether the iron be in intimate organic combination, the so called food iron, incapable of giving the characteristic microchemical reaction, or whether it be in the form of an organic or inorganic salt of iron. Moreover, from the work of Häusermann (2) and of Abderhalden (3), it appears that though iron salts are absorbed, the body is unable, or but very poorly able, to utilize them for the building of hemoglobin, being dependent for this constructive work upon the intimately combined food iron. On the other hand, iron salts are effective stimulants to the blood-

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[1] *Jour. Exper. Med.*, 1912, xvi, 363, 375, 758, 769, 780; 1913, xviii, 487, 494, 665; 1914, xx, 19, 108.

forming organs and conspicuously increase the utilization by them of the food iron.

The elimination of iron occurs almost wholly through the intestines, especially the colon, the quantity passing out in the urine constituting less than 1 per cent. of the total excretion in man and the dog. In the fasting dog the output found by von Voit (4) was 0.60 of a milligram per kilo of body-weight per day, and on an adequate, but iron-poor diet, Gottlieb's (5) dog excreted 0.34 of a milligram. For man the figures are lower. Cetti and Breithaupt (6), while fasting, eliminated about 0.10 to 0.13 of a milligram per kilo per day; and in various studies on man 0.10 to 0.25 of a milligram per kilo per day have been found to be the intake required to maintain iron equilibrium. However, there is every reason to believe, as is suggested by the work of Schmidt (7), who fed mice for months on a diet extremely poor in iron, but obtained no fall in the hemoglobin, that the organism possesses great power of conserving its iron and of reutilizing it through some form of intermediary metabolism. When, however, Schmidt withdrew iron from the diet for several generations, the younger generations were extremely anemic and this anemia disappeared upon restoring iron to the diet. As the iron-poor diet led to the disappearance of microchemically demonstrable iron from the liver, but affected to a much slighter degree that of the spleen, Schmidt concluded that the liver is the depot for iron from the food, and that the spleen, on the other hand, is the depot for iron from tissue and erythrocyte catabolism and thus an important factor in the intermediary metabolism of iron.

If the spleen plays this part in iron metabolism, its absence might well interfere with the reutilization of iron by the organism and lead to an increased iron elimination, and this Asher and his coworkers, Grossenbacher and Zimmermann, claim to have demonstrated in dogs. They studied the iron elimination of four puppies from two litters; one from each litter was splenectomized and one from each was kept as a control. The iron estimations were made at intervals of a few weeks, two months, and ten months after splenectomy, and in all their experiments they found an output much higher, often double, in the splenectomized animals as compared with the controls.

Bayer (8) has made some studies on man of the iron elimination following splenectomy for rupture of the spleen or for Banti's disease, and has compared the output for a certain number of days on known diets with that of control cases on the same diets. His results are summarized in the following table.

TABLE I.

Disease.	Age in yrs.	Time after splenectomy.	Output of iron in mg. per kilo per dy.	
			Splenectomized.	Control.
Spleen rupture..........	16	2 wks.	0.22	0.16
			0.15	0.18[2]
Spleen rupture..........	16	3 wks.	0.30	0.22
			0.17	0.08
			0.51	0.50
Banti's disease..........	25	6 mos.	0.19	0.18[2]
Banti's disease..........	19	2 yrs.	0.19	0.18[2]

From these experiments the author concludes that there is an increased output of iron soon after splenectomy, as shown by the second observation in the table, but that later the elimination returns to normal. Bayer's statement that certain of his diets contained 0.24 of a gram of iron per day is probably an error since a diet of the general character that he describes would certainly have a much lower iron content.

METHODS.

In our earlier experiments we studied the iron elimination during four-day periods, but found that these periods led to irregular results. In the work here reported, therefore, we present only observations based on periods of nine or ten days' duration.

The animals were placed in metabolism cages with glass floors and after they had been fed for several days on constant weighed amounts of the diet selected, the rectum was emptied by the use of morphin; iron-free charcoal was added to the next feeding, and the collection of feces was begun from the appearance of the charcoal; at the close of the period the rectum was again emptied with morphin, carmine was added to the next feeding, and the feces were collected until carmine appeared in them. In the earlier experiments the urine also was analyzed, but as only traces of iron, less than 1 per cent. of the total elimination, were found the urine was omitted in our later analyses. To avoid the introduction of extraneous iron, the feces were collected by means of a nickel spatula soon after being passed.

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[2] These three figures are merely repetitions of a single control experiment.

In one group of experiments representing the earlier periods after operation, we have studied the output of iron on the same dogs, both before and after splenectomy, without a change in diet. In another group, representing later periods, we have compared the output of normal control dogs with that of splenectomized dogs of approximately the same weight on corresponding diets.

The analyses were made by the method of Ripper and Schwarzer (9), slightly modified. The feces collected for the entire period are placed in a quartz dish, dried, and ashed dry. The ash is extracted with boiling concentrated hydrochloric acid and filtered, and the residue washed with 20 per cent. hydrochloric acid. The residue and filter paper are re-ashed and the extraction is repeated. This ashing and extraction is continued until the extract ceases to give a positive test with potassium sulphocyanide.

The total filtrate is made up to a known volume and two duplicate portions, containing presumably two to five milligrams of iron, are taken. To each is added one cubic centimeter of hydrogen peroxide (Merck's Blue label), and the solution evaporated to dryness on a water bath. The residue is then redissolved in one cubic centimeter of 20 per cent. hydrochloric acid and twenty cubic centimeters of boiling water are used in four small portions, and then this washing with acid and water is repeated. In the course of the manipulation the entire solution is brought into a 200 cubic centimeter Erlenmeyer flask.

All the specimens to be analyzed at one time having been brought to this stage, a standard is prepared by placing into each of two 200 cubic centimeter Erlenmeyer flasks forty cubic centimeters of a quantitative ferric chloride solution containing about 0.002 of a gram of iron. To each of the flasks, those containing the specimens and the two containing the standard, there are added in rapid succession four grams of potassium iodide; the flasks are then immediately stoppered and placed in a water bath at 60° C. for ten minutes. At the end of this time the flasks are removed, and to each 100 cubic centimeters of cold water are immediately added and the flask is restoppered.

To each flask in turn is added starch solution, and the contents are titrated with sodium thiosulphate solution, approximately N/250, until disappearance of the blue color, and then they are immediately titrated with weak iodin solution back to the first reappearance of the blue color. In each analysis the thiosulphate solution is freshly prepared and standardized against the two flasks of known ferric chloride solution, and the iodin solution also is freshly prepared and standardized against the thiosulphate solution. The precision of the titration method is found to be greatly enhanced by the titration back with iodin to the first reappearance of the blue color and calculation accordingly of the thiosulphate end point.

In control experiments performed by adding known amounts of iron to one of identical pairs of samples of ash of feces, an error of about 2 per cent. was observed.

The food used in these experiments consisted of casein, cracker meal, lard, and fresh beef heart in proportions designed to give the desired amount of iron. The iron content of the food was determined by analyzing many large portions (each 50 to 400 grams) of the beef heart, cracker, and casein and obtaining average figures for use in calculating the iron content of the diets employed.

RESULTS.

In the accompanying tables are given in detail the final figures obtained in our studies. The experiments are divided into two groups. First, five animals were studied both before and for two weeks after splenectomy, on a constant diet throughout; these are arranged in table II according to the iron content of the diet. Second, a group of six animals (table III), three normal controls and three splenectomized animals, were studied at longer periods after splenectomy; these were of about the same weight and were on diets of the same general character, but varying in the content of iron.

Inspection of table II shows that the iron output of dogs 88 and

35 is unchanged by splenectomy, but that dogs 30, 44, and 79 show some increase. On the other hand, in table III, it will be seen that all three splenectomized dogs exhibit an output of iron as compared with the intake closely comparable with that of the controls. From our studies it would appear therefore that during the first two weeks after splenectomy, some, but not all dogs show a slight increase in the output of iron, but that at 1 month, 9 months, and 20 months after splenectomy we find no indication of an increased iron output. The occasional evanescent and inconstant increase in elimination of iron does not justify the conclusion that the spleen exerts an important influence on iron metabolism. Our results are obviously different from those of Asher and his associates, and as a possible explanation of this we would call attention to the extreme shortness of the periods—one to three days—employed

TABLE II.

Dog No.	Average weight.	Duration of periods.	Intake.[3]	Output.[3]		Time after splenectomy.
				Before splenectomy.	After splenectomy.	
88	7,000	10 dys.	0.27	0.67	0.70	4–14 dys.
30	5,340	9 dys.	0.30	0.36	0.55	1–10 dys.
35	7,720	9 dys.	0.64	0.87	0.81	1–10 dys.
44	9,000	9 dys.	1.57	1.89	2.10	1–10 dys.
79	9,000	9 dys.	1.71	1.88	2.21	6–15 dys.

TABLE III.

Dog No.	Controls.				Time after splenectomy.
	Average weight.	Duration of period.	Intake.[4]	Output.[4]	
79	9,000	9 dys.	1.00	1.42	
44	9,000	9 dys.	1.57	1.89	
79	9,000	9 dys.	1.71	1.88	
		Splenectomized.			
83	8,400	10 dys.	1.42	1.39	27–37 dys.
9	8,800	9 dys.	1.35	1.56	9 mos.
51	10,000	9 dys.	1.32	1.42	20 mos.

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[3] Figures expressing intake and output indicate milligrams of iron per kilo per day.

[4] Figures expressing intake and output indicate milligrams of iron per kilo per day.

by Asher and Grossenbacher, and to their failure to mark in any way the stools. In the studies of output ten months after splenectomy, as given by Asher and Zimmermann, the splenectomized dog in most of the experiments was much larger than the control, so that if the iron output of their dogs be calculated per kilo of body-weight it will be found that the output of the splenectomized animals approaches very closely that of the normal controls, and is in some instances identical. It seems possible that in these studies ten months after splenectomy the increased iron output of the splenectomized animals was due rather to the size of the animal than to the splenectomy, and it is doubtful, therefore, whether the conclusions of Asher and Zimmermann, based on these experiments, are justified.

CONCLUSIONS.

Our studies give evidence of increase in the iron elimination in three of five dogs during a period of two weeks following splenectomy, but not in two other dogs. The occasional increased output of iron may have some relation to the anemia which occurs in the early weeks after splenectomy and which varies in degree in different animals.

No evidence was secured of an increase in the iron output at 1, 9, and 20 months after splenectomy.

From our own studies and from examination of the literature of the subject, we conclude that the spleen does not exercise a constant and important influence upon the iron metabolism of the body.

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PARAMENINGOCOCCUS AND ITS ANTISERUM.*

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In the course of the treatment of epidemic meningitis with the antimeningococcic serum it became evident that a proportion of cases of the disease treated with the serum failed to react favorably. In some of these cases the meningococcus contained in the cerebrospinal exudate survived, continued to multiply, and failed to be phagocyted by the leucocytes, in contradistinction to what happened in the majority of instances. That the meningococci occurring in the resistant cases were in part resistant, or fast, to the antiserum was suspected, and two possible kinds of fast strains were recognized: first, strains originally fast at the time the serum treatment was begun; and, second, strains developing fastness in the course of the serum treatment.[1] The second group embraced those instances in which meningococci at first reacted to the serum but later failed to do so, leading to a relapse which continued to a fatal termination.

A more precise definition of the fast strains has not thus far been made. However, Dopter[2] has studied a special class of the cocci obtained from the nasal secretion and called by him parameningococci, which while resembling the common meningococcus in fermentative and cultural properties differs from it in certain immunological reactions. The parameningococcus, so called, has now been found by Dopter and other French writers to invade the meninges and the blood and is believed by them to be one of the causes of cerebrospinal meningitis.[3] According to the French ob-

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[1] Flexner, S., *Jour. Exper. Med.*, 1913, xvii, 553.

[2] Dopter, Ch., *Compt. rend. Soc. de biol.*, 1909, lxvii, 74.

[3] The literature has been reviewed by Dr. Flexner in his article on antimeningococcic serum (Flexner, S., in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, 2d edition, Jena, 1914 (in press)).

servers, when acute meningitis arises through the presence of the parameningococcus it is not controllable by means of the ordinary antimeningococcic serum, but does respond to a special antiserum prepared with cultures of the parameningococcus.

The subject of the atypical strains of the meningococcus is an important one since it affects the question of a better control of cases of epidemic meningitis by means of a serum containing antibodies for the unusual varieties. On that account we have made a study of two cultures of the parameningococcus obtained from Dr. Dopter through the kindness of Dr. Louise Pearce, who made a hurried journey to Paris to secure them, since it was found that the cultures sent by post did not survive the journey.

IMMUNITY REACTIONS OF THE MENINGOCOCCUS AND PARAMENINGOCOCCUS.

The meningococcus is subject to the several immunity reactions of agglutination, complement deviation, and opsonization. All the reactions are specific, although certain strains react not at all or imperfectly. As regards agglutination, it may be said that considerable variations arise affecting strains of meningococci relatively or absolutely inagglutinable. And yet agglutination is perhaps the most trustworthy guide in the identification of the meningococcus through specific serum reactions.[4] While immune sera may possess high agglutinating value for meningococci, normal sera possess either none, or very little; the inagglutinable strains react to immune sera of high value no more than they do to normal sera.

On the one hand, the inagglutinable strains react differently to a given meningococcic serum, while again a given agglutinable strain may react unequally to immune sera prepared from different strains of meningococci.[5] It should further be noted that while homologous sera tend to agglutinate corresponding strains best, exceptions occur in which they agglutinate heterologous strains better.[5,6]

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[4] Flexner, S., in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, *loc. cit.* Elser, W. J., and Huntoon, F. M., *Jour. Med. Research,* 1909, xx, 371.

[5] Eberle, J., *Arch. f. Hyg.,* 1908, lxiv, 171.

[6] St. Baecher, in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, Jena, 1911, supplement 1, 80.

Notwithstanding the variations indicated, it is obviously still desirable to determine the degree in which parameningococci differ from normal meningococci in respect to immunity reactions, and to ascertain whether they form a special class or merely constitute variations from the normal type. For this purpose two cultures, L and M, of the parameningococcus secured from Dr. Dopter have been subjected to the reactions of agglutination, complement deviation, and opsonization, respecting which they have been compared with several strains of normal meningococci. The immune sera employed consisted of a polyvalent antimeningococcic serum prepared by the Department of Health of the City of New York, and several monovalent sera produced in the rabbit by immunization with single strains of the microörganisms.

It will conduce to clearness and simplicity of presentation to describe briefly the different strains of meningococci which were studied.

The parameningococci consisted of Gram-negative diplococci indistinguishable from ordinary meningococci in form, staining properties, and fermentative reactions. They were also subject to autolysis in the manner of normal meningococci.

Twenty other strains of meningococci were employed for comparison. Four, HP, MA, B, and 138, came from the Pasteur Institute, having been secured by Dr. Pearce along with the parameningococci. They were regarded as normal strains. It may be noted here that all four exhibited irregularities of agglutination, and B proved inagglutinable.

One strain, W, came from Great Britain in 1908, and was isolated from a case of posterior basic meningitis. It was a normal strain. The remaining fifteen strains were obtained in New York, partly from the stock of The Rockefeller Institute, partly from the Department of Health.[7] They have been classified as follows: Eight are normal strains agglutinating regularly. They are designated F, 20, 25, 28, 35, 45, 48, and 49. Cultures 25 and 48 were derived from rapidly fatal fulminating cases of meningitis; 28 is from a severe case terminating fatally on the fourth day; 45 is from a fatal case developing basic symptoms; 35 is from a mild case becoming chronic, in which hydrocephalus developed before death; and 49 is from a mild case which recovered.

Five are normal strains agglutinating irregularly. BH was derived from a fatal case in an infant; 9 and 18 were without history; and 37 was obtained from a case which recovered under serum treatment. Two strains, 7 and 42, gave agglutination reactions similar to the parameningococci, and had been employed in the manufacture of the antimeningococcic serum by the New York Department of Health. Table I furnishes a means of ready reference.

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[7] For the Board of Health cultures I am indebted to Dr. Phoebe Du Bois and Dr. Marie Grund.

TABLE I.

Source of Cultures.

Designation.	Source.	Nature of strain.	Type of agglutination.
L	Dopter	Para	
M	Dopter	Para	
HP	Pasteur Institute	Normal	Irregular.
MA	Pasteur Institute	Normal	Irregular.
138	Pasteur Institute	Normal	Irregular.
B	Pasteur Institute	Normal	Inagglutinable.
W	Great Britain	Normal	Regular.
F	New York	Normal	Regular.
18	New York	Normal	Regular.
25	New York	Normal	Regular.
28	New York	Normal	Regular.
35	New York	Normal	Regular.
45	New York	Normal	Regular.
48	New York	Normal	Regular.
49	New York	Normal	Regular.
BH	New York	Normal	Irregular.
I	New York	Normal	Irregular.
9	New York	Normal	Irregular.
20	New York	Normal	Irregular.
37	New York	Normal	Irregular.
7	New York	Normal	Para-like.
42	New York	Normal	Para-like.

Besides the results of agglutination, other immunity reactions were studied; those, namely, of opsonization, complement deviation, and protection. These reactions are subject also to irregularities and variations, and notably that of complement deviation,[8] which has been generally given up as a method of determining the value of the antimeningococcic serum in therapeutic immunity principles.

AGGLUTINATION.

Several immune sera were employed for determining the immunity reactions. One, that of the New York Health Department, was prepared in the horse from many strains of meningococcus and preserved with 0.3 per cent. tricresol. The monovalent sera were made by immunizing rabbits with single strains. The inoculations were conducted over periods of several months. For the general

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[8] Flexner, S., in Kraus, R., and Levaditi, C., Handbuch der Technik und Methodik der Immunitätsforschung, *loc. cit.*

TABLE II.

Monovalent Rabbit Serum Immune to Parameningococcus L. Agglutination Reactions Made at 55° C.

Strains.	C	10	20	50	100	200	500
Para L	–	++	++	++	++	++	+
Para M	–	++	++	+	+	+	–
48	–	+	+	±	–	–	–
45	–	+	–	–	–	–	–
49	–	++	+	±	–	–	–
42	–	++	++	++	+	+	–
35	–	++	–	–	–	–	–
37	–	+	+	+	+	–	–
138	–	+	+	±	–	–	–
28	–	++	±	–	–	–	–
25	–	++	++	+	±	–	–
20	–	++	++	++	+	±	–
18	–	++	++	++	+	±	–
9	–	++	++	+	±	–	–
7	–	++	++	+	+	–	–
B	–	–	–	–	–	–	–
MA	–	+	+	+	–	–	–
HP	–	+	+	+	–	–	–
W	–	++	+	+	±	–	–
I	–	+	+	+	–	–	–
BH	–	+	+	+	+	–	–
F	–	+	+	–	–	–	–

TABLE III.

Monovalent Rabbit Serum Immune to Parameningococcus M. Agglutination Reactions Made at 55° C.

Strains.	C	10	20	50	100	200	500
Para M	–	++	++	++	++	+	+
Para L	–	++	++	+	+	±	–
48	–	+	±	–	–	–	–
45	–	+	+	+	–	–	–
49	–	++	++	+	±	–	–
42	–	++	++	++	+	+	–
35	–	+	±	–	–	–	–
37	–	++	++	+	±	–	–
138	–	+	+	+	–	–	–
28	–	+	+	–	–	–	–
25	–	+	+	±	–	–	–
20	–	++	++	++	+	–	–
18	–	++	++	++	+	–	–
9	–	++	++	++	+	–	–
7	–	++	++	++	+	+	–
B	–	+	–	–	–	–	–
MA	–	+	+	+	+	±	–
HP	–	+	+	+	+	+	–
W	–	++	+	±	–	–	–
I	–	++	++	++	+	+	–
BH	–	+	+	+	+	+	–
F	–	+	+	±	–	–	–

work the two Dopter strains of parameningococci and normal strains 35 (mild case), 45 (basic case), and 48 (fulminating case) were used.

At the expiration of three months the titer of the rabbit sera did not exceed 1 to 500. But as normal rabbit serum is inactive in dilutions greater than 1 to 10, the specific effects could be readily followed. A few exceptions with normal strains occurred. Thus strains 45 and 138 agglutinated in 1 to 20, and strain 37 in 1 to 50. Normal horse serum is inactive above 1 to 20. Here again certain normal strains, namely 25, 45, and 138, were somewhat more sensitive and reacted in 1 to 50 to 1 to 100 dilutions.

Parameningococcus.—The polyvalent antimeningococcic horse serum was almost wholly inactive upon the two Dopter strains of parameningococci, while it agglutinated the two para-like strains 7 and 42 in dilutions 1 to 200. It should be recalled that the two latter strains were employed in the preparation of the serum.

The monovalent parameningococcus rabbit sera exhibited varying titers according as they acted upon the homologous or heterologous strains. With the former the limit was 1 to 500, with the latter 1 to 200. No difference was noted between the sera prepared from strains L or M (tables II and III).

On the other hand, the two para-like strains, 7 and 42, gave slightly different reactions according to the source of the immune parameningococcus serum. With para serum L, strain 42 agglutinated at 1 to 200, and strain 7 at 1 to 100; with para serum M, both agglutinated at 1 to 200. In other words, strains 7 and 42 behave as heterologous para strains against these two sera.

If we turn now to a monovalent serum prepared from normal strain 48 which agglutinated its own and two other normal strains in 1 to 500, the two Dopter para strains, L and M, agglutinated in 1 to 20, and the two para-like strains at 1 to 20 (strain 42) and 1 to 50 (strain 7) (table IV).

However, the Dopter para sera are not without agglutinating effects on normal strains of meningococcus, and both those that agglutinate regularly and irregularly. With para serum L, among the former, strain 18 reacts in 1 to 100, strains 25 and W in 1 to 50 dilutions; among the latter, strains 37 and BH react in 1 to 100, strain 20 in 1 to 100 dilutions. With para serum M regular strains 18 and 49 react in dilutions of 1 to 100 and 1 to 50, respectively, irregular strains HP, I, and BH react in 1 to 200, and strains 9 and 20 in 1 to 100 dilutions.

TABLE IV.

Monovalent Rabbit Serum Immune to Meningococcus 48. Agglutination Reactions Made at 55° C.

Strains.	C	10	20	50	100	200	500
48	–	++	++	++	++	+	+
Para M	–	++	+	±	–	–	–
Para L	–	++	+	–	–	–	–
45	–	+	+	+	+	–	–
42	–	+	+	–	–	–	–
49	–	++	++	++	+	–	–
35	–	++	++	++	++	+	+
37	±	++	++	++	++	+	–
138	–	++	++	++	++	+	–
28	–	++	++	+	–	–	–
25	–	++	++	++	++	++	+
20	–	++	+	+	–	–	–
18	–	++	++	++	++	+	±
9	–	++	++	+	±	–	–
7	–	++	++	±	–	–	–
B	–	+	+	±	–	–	–
MA	–	+	+	+	+	–	–
HP	–	+	+	+	+	+	–
W	–	++	++	++	+	±	–
I	–	++	+	+	±	–	–
BH	–	++	+	+	+	+	–
F	–	++	++	++	+	–	–

The conclusion to be drawn from this series of tests is not to the effect that parameningococcus strains are strictly different as regards agglutination from normal strains of meningococcus, but that they nevertheless display a certain relative specificity.

Normal Meningococcus.—Two classes of normal meningococci have been recognized. They have been denominated "regular" and "irregular" according as they agglutinate in all or only in part of the normal immune sera. The variations in regard to agglutinability among normal strains are wide, as is exhibited in table V. It is this great variability that makes it impracticable on the basis of agglutination alone to separate certain strains as being a distinct group or species. It remains true, however, that the group distinguished by the name of "para" departs even more widely from the normal standard than do the several irregular strains studied. And this difference reappears in respect to other immune reactions to be described.

TABLE V.

Agglutination Reaction at 55° C. Limit Dilutions for Complete Agglutination.

Strain.	Sera.				
	Board of Health.	Para M.	Para L.	Normal 48.	Normal 35.
Para L	1 : 10	1 : 100	1 : 500	1 : 20	1 : 10
Para M	1 : 10	1 : 500	1 : 200	1 : 20	1 : 20
Para-like 7	1 : 200	1 : 200	1 : 100	1 : 20	1 : 50
Para-like 42	1 : 100	1 : 200	1 : 200	1 : 20	1 : 50
Normal regular W	1 : 500	1 : 20	1 : 50	1 : 100	1 : 100
Normal regular F	1 : 50	1 : 20	1 : 20	1 : 100	1 : 50
Normal regular 18	1 : 50	1 : 100	1 : 100	1 : 200	1 : 100
Normal regular 25	1 : 50	1 : 20	1 : 50	1 : 500	1 : 200
Normal regular 28	1 : 100	1 : 20	1 : 10	1 : 50	1 : 50
Normal regular 35	1 : 10	1 : 10	1 : 10	1 : 500	1 : 500
Normal regular 45	1 : 50	1 : 50	1 : 10	1 : 100	1 : 200
Normal regular 48	1 : 50	1 : 10	1 : 20	1 : 500	1 : 50
Normal regular 49	1 : 100	1 : 50	1 : 20	1 : 100	1 : 100
Normal irregular BH	1 : 500	1 : 200	1 : 100	1 : 200	1 : 200
Normal irregular I	1 : 100	1 : 200	1 : 50	1 : 50	1 : 100
Normal irregular 9	1 : 200	1 : 100	1 : 50	1 : 50	1 : 50
Normal irregular 20	1 : 50	1 : 100	1 : 100	1 : 50	1 : 100
Normal irregular 37	1 : 100	1 : 50	1 : 100	1 : 200	1 : 100
Normal irregular 138	1 : 200	1 : 50	1 : 20	1 : 200	1 : 50
Normal irregular HP	1 : 20	1 : 200	1 : 50	1 : 200	1 : 200
Normal irregular MA	1 : 200	1 : 100	1 : 50	1 : 100	1 : 100
Inagglutinable B	1 : 20	1 : 10	0	1 : 20	0

Hence it is apparent that a clean cut classification into parameningococcus and meningococcus strains has been possible with thirteen only of the twenty-two strains whose agglutination reactions have been studied. The remaining nine act either irregularly or so nearly alike in all the sera tested that definite discrimination is not possible.

Attention is directed also to the fact that lack of agglutination by polyvalent antimeningococcic horse serum is insufficient evidence for the classification of meningococci into para and normal strains, since even normal meningococci do not invariably agglutinate in such a serum in high dilution, and some strains fail to agglutinate in dilutions greater than 1 to 20.

OPSONINS AND COMPLEMENT DEVIATION.

Opsonins.—The opsonin content is employed extensively for determining the therapeutic value of antimeningococcic serum. It was desirable therefore to test the specificity of this reaction upon normal and para strains of meningococci. For this purpose the Neufeld technique was employed. The result is shown in table VI, which tends again to isolate the two parameningococcus strains of Dopter from the strains of normal meningococcus employed.

TABLE VI.

Opsonization.

Serum.	Strain.	Control.	1:10	1:20	1:50	1:100	1:200	1:500	1:1,000	1:2,000	1:5,000
Board of Health	Para L	–	+	–	–	–	–	–	–	–	–
	Para M	–	+	–	–	–	–	–	–	–	–
	48	–	+	++	+	+	+	±	–	–	–
	35	±	++	+	+	±	–	–	–	–	–
	I	–	++	++	++	++	+	+	+	+	±
Fulminating case	48	–	+	+	+	+	+	±	–	–	–
	35	–	++	++	++	+	+	±	–	–	–
	Para L	–	+	+	+	–	–	–	–	–	–
	Para M	–	+	+	–	–	–	–	–	–	–
	I	–	+	+	+	±	–	–	–	–	–
Para M	Para M	–	+	+	+	+	+	+	–	–	–
	Para L	–	+	+	+	+	–	–	–	–	–
	48	–	+	+	±	–	–	–	–	–	–
	I	–	+	+	±	–	–	–	–	–	–
Para L	Para L	–	+	+	+	+	+	±	–	–	–
	Para M	–	+	+	+	+	–	–	–	–	–
	48	–	+	+	+	–	–	–	–	–	–
	I	–	+	±	–	–	–	–	–	–	–
Mild case	35	–	++	++	++	++	+	±	–	–	–
	48	–	++	++	+	+	±	–	–	–	–
	Para L	–	+	+	–	–	–	–	–	–	–
	Para M	–	+	+	–	–	–	–	–	–	–

Complement Deviation.—The degree of deviation of complement exerted by an antimeningococcic serum was recommended by Kolle and Wassermann to estimate its therapeutic value. Since, however, it appears that the reaction is subject to considerable and unexplained fluctuations it has not been generally adopted. None the less, it was desirable to determine the degree of specificity of the reaction as applied to para and normal strains.

Antigens were prepared by the method of Schwartz and McNeil.[9] When the antigens made with the parameningococci of Dopter were tested against the polyvalent antimeningococcic horse serum, complement was bound. When, however, the monovalent parameningococcic sera were titrated against normal meningococci, complement deviation occurred only in low dilutions. When the parameningococci antigens were titrated against monovalent sera prepared with normal meningococci strains 48 and 35, no binding was obtained. On the other hand, antigens of strains 48 and 35 deviated complement of the homologous sera in low dilutions (table VII). The action of the polyvalent antimeningococci serum corresponds with the results obtained by Dopter,[10] who, however, also noted that the sera of patients suffering from parameningococcal infection deviated complement in the presence of parameningococci but not of normal meningococci. If this is strictly true, then it must be held that the monovalent rabbit sera are less specific than the serum of patients.

The conclusion to be drawn from the tests of complement deviation is again to the effect that the para strains deviate from the normal strains, although the distinction cannot be said to be absolute.

PROTECTION EXPERIMENTS.

In the end the existence of profound differences between the para and normal strains of meningococci will be determined not so much by the immunity reactions already described, as by the results of protection tests, since in practice it is this test that determines whether special account should be taken of the para strains in the preparation of the antimeningococcic serum. For the purpose of the protection tests guinea pigs and monkeys were employed.

That guinea pigs weighing about 125 grams are especially susceptible to inoculation with cultures of the meningococcus was noted by Flexner.[11] I am able to confirm his observation. The tests were conducted with lethal doses by intraperitoneal injection of the

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[9] Schwartz, H. J., and McNeil, A., *Am. Jour. Med. Sc.*, 1912, cxliv, 815.

[10] Dopter, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1911, xxxi, series 3, 590.

[11] Flexner, S., *Jour. Exper. Med.*, 1907, ix, 105.

TABLE VII.

Complement Deviation.

Complement, guinea pig serum, 1:40 dilution, in c.c.	Immune rabbit serum Para M, in c.c.	Para M antigen, in c.c.	Sheep corpuscles, 1:20 dilution, in c.c.	Anti-sheep rabbit serum, 1:100 dilution, in c.c.	Result.
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.2	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	No hemolysis.
		Culture 48 antigen.			
0.1	0.1	0.25	0.1	0.1	Incomplete hemolysis.
0.1	0.2	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	Complete hemolysis.
	Immune serum, fulminating case.				
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	No hemolysis.
0.1	0.01	0.25	0.1	0.1	No hemolysis.
		Culture 35 antigen.			
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	No hemolysis.
0.1	0.01	0.25	0.1	0.1	No hemolysis.
		Para L antigen.			
0.1	0.1	0.25	0.1	0.1	Complete hemolysis.
0.1	0.1	0.15	0.1	0.1	Complete hemolysis.
0.1	0.01	0.25	0.1	0.1	Complete hemolysis.
		Para M antigen.			
0.1	0.1	0.25	0.1	0.1	Complete hemolysis.
0.1	0.1	0.15	0.1	0.1	Complete hemolysis.
0.1	0.01	0.25	0.1	0.1	Complete hemolysis.
	Board of Health serum.				
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	Complete hemolysis.
0.1	0.01	0.25	0.1	0.1	Complete hemolysis.
		Para L antigen.			
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	No hemolysis.
0.1	0.01	0.25	0.1	0.1	Complete hemolysis.
		Culture 48 antigen.			
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	Incomplete hemolysis
0.1	0.01	0.25	0.1	0.1	Incomplete hemolysis.
		Culture 35 antigen.			
0.1	0.1	0.25	0.1	0.1	No hemolysis.
0.1	0.1	0.15	0.1	0.1	No hemolysis.
0.1	0.01	0.25	0.1	0.1	Complete hemolysis.

several cultures alone and combined with homologous and heterologous sera. Preliminary experiments were made to determine the effects of normal rabbit and horse serum. They were found not to protect in corresponding doses against the cultures used. The cultures alone in the doses given invariably caused death. The following is a detailed example of a protective experiment in young guinea pigs, of which table VIII presents the results in brief.

Experiment 1.—A. May 27, 1914. Two guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M.

May 28. Both guinea pigs were dead.

B. May 27. Four guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M, followed at once by 0.5 c.c. of Para M immune rabbit serum.

May 28. Four guinea pigs were living but one was ill.

May 29. One guinea pig was dead.

C. May 27. Four guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M, followed at once by 0.5 c.c. of Para L immune rabbit serum.

May 28. All four guinea pigs were dead.

D. May 27. Four guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M, followed at once by 0.5 c.c. of immune rabbit serum 48.

May 29. Three guinea pigs were dead.

E. May 27. Four guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M, followed at once by 0.5 c.c. of immune rabbit serum 35.

May 28. All four guinea pigs were dead.

F. May 27. Four guinea pigs weighing 120 gm. each were injected intraperitoneally with 0.2 c.c. of a 24-hour old sheep serum agar culture of Para M, followed at once by 0.5 c.c. of Board of Health antimeningitis serum.

May 28. One guinea pig was dead.

What is noticeable is the general fluctuation of protection in that each monovalent serum, while being most perfectly protective for its homologous organism, exerts, also, some, if variable, amounts of protection against other or heterologous organisms. In this regard it cannot be said that the para strains of Dopter acted more regularly and specifically than the normal strains. On the whole, and as was probably to be expected, the Board of Health polyvalent serum showed the greatest regularity of action. In other words, this polyvalent serum carried protective immune bodies in about equal amount for the normal and para strains.

It was deemed desirable to ascertain the protective value of mono-

TABLE VIII.

Serum Protection Experiments.

Serum.	Cultures employed.				
	Para L.	Para M.	Culture 48.	Culture 35.	Culture 45.
Para L	Protected all	Protected none	Protected 8 of 10	Protected all	Protected none.
Para M	Protected 4 of 10	Protected 7 of 10	Protected 5 of 10	Protected none	Protected none.
Culture 48	Protected 6 of 10	Protected 4 of 10	Protected all	Protected 9 of 10	Protected none.
Culture 35	Protected 4 of 8	Protected none	Protected 5 of 6	Protected all	Protected none.
Board of Health	Protected 5 of 6	Protected 5 of 6	Protected 5 of 6	Protected 3 of 6	
Normal horse	Protected none	Protected none	Protected none	Protected none	
Normal rabbit	Protected none	Protected none	Protected none	Protected none	

valent sera upon monkeys infected by intraspinal inoculation and treated in the same manner. For this purpose parameningococcus L (Dopter) was employed for infection. The culture proved to be of low virulence, necessitating large doses in order to set up fatal infection. The method was to inject the culture and then immediately afterwards the immune sera. The following small series of experiments was made.

Experiment 1.—Control. A *Macacus rhesus* received intraspinally the surface growths of two sheep serum water agar slant cultures suspended in normal saline. Three hours after the injection the animal became ill; death occurred in twenty hours. At autopsy the meninges were congested and edematous; cultures of parameningococcus L were recovered.

Experiment 2.—A second *Macacus rhesus,* having received a similar dose of the suspended culture, was given five minutes later 1.5 c.c. of parameningococcus rabbit serum L. Slight symptoms of illness only developed. Twenty-four hours later lumbar puncture yielded turbid fluid containing polynuclear leucocytes enclosing diplococci; no diplococci were found outside of cells. A second dose of 1.5 c.c. of the immune serum was administered. At the expiration of another twenty-four hours the animal appeared well and the cerebrospinal fluid was clear.

Experiment 3.—A third *Macacus rhesus* was inoculated with the established dose of parameningococcus L and five minutes later was given 1.5 c.c. of immune rabbit serum prepared from normal meningococcus 48. No protection was afforded, and death occurred within twenty hours.

Experiment 4.—The fourth and last test was made with parameningococcus L and immune rabbit serum prepared with parameningococcus M. It was a repetition of experiment 2. The animal recovered completely.

The series of tests with monkeys is of value in supporting the group distinction between the normal and para meningococci. It is highly improbable that in a larger series of experiments some degree of cross-protection should not have been found between the normal and para organisms. On the other hand, the experiments indicate that para sera L and M are equally effective for protection against a given parameningococcus as in the case of para organism L.

DISCUSSION.

The study of *Diplococcus intracellularis* or meningococcus and allied organisms has led to the setting up of four classes as follows: (1) pseudomeningococci found by von Lingelsheim;[12] (2) diplococci derived from cases of posterior basilar meningitis described by Houston[13] and other English workers; (3) S strains isolated by Friese and Müller[14] from the nasopharynx of patients not having meningitis, and classified by Sachs-Müke[15] as pseudomeningococci; and finally (4) the diplococci described by Dopter as parameningococci. Von Lingelsheim's cocci are so readily differentiated from true meningococci by their morphological and cultural characteristics that they require no further mention. The other three classes, however, are described as being morphologically and culturally indistinguishable from true meningococcus, differing only in serum reactions, especial stress being laid upon differences in agglutinating power.

The diplococci from cases of basilar meningitis have been shown to be true meningococci,[16] a fact further substantiated by the tests made in the present study with diplococci from two personal cases of chronic basilar meningitis, and by one strain sent by Dr. Houston in 1908.

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[12] von Lingelsheim, W., *Klin. Jahrb.*, 1906, xv, 373.

[13] Houston, T., and Rankin, J. C., *Brit. Med. Jour.*, 1907, ii, 1414.

[14] Friese, H., and Müller, H., *Klin. Jahrb.*, 1909, xx, 321.

[15] Sachs-Müke, *Klin. Jahrb.*, 1911, xxiv, 425.

[16] Wollstein, M., *Jour. Exper. Med.*, 1909, xi, 579.

The S cocci described by Friese and Müller were not obtained from meningitis patients nor from persons who had been in contact with cases of meningitis, and all the cultures differed markedly in agglutination reactions from strains of true meningococcus. In the absence of other serum tests it is not possible to bring these cocci into relation with parameningococci.

Dopter's parameningococci remain, then, in a class by themselves, differing serologically more or less from other diplococci. Although Dopter first found them in the nasal mucus of persons who had been in contact with meningitis patients, other observers soon demonstrated their presence in the blood and cerebrospinal fluid. Thus in 1910 a case of purpura fulminans with septicemia was observed by Carnot and Marie,[17] from which the organism was isolated from the blood. No meningitis was present in this case. Menetrier[18] was the first to report a case of meningitis due to parameningococcus. The patient was an infant and Menetrier noted a marked difference between the apparent mildness of the symptoms and the intensity of the infection as evidenced by the character of the cerebrospinal fluid, in which the majority of the cocci were extracellular. Injections of the usual antimeningococcic serum instead of causing amelioration of symptoms and fall in temperature were followed by intensification of symptoms. Seven cases of meningitis due to the parameningococcus having come under Dopter's observation, he recommended the therapeutic use of ordinary antimeningococcic serum to be followed later by an antiparameningococcic serum if laboratory examination showed the presence of parameningococcus infection. Dopter[19] prepared such an antiparameningococcus serum in 1912, and its use was followed by the recovery of cases of meningitis caused by the parameningococcus

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[17] Carnot and Marie, P.-L., *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1911, xxxi, series 3, 74.

[18] Menetrier, *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1911, xxxi, series 3, 82.

[19] Dopter, *Semaine méd.*, 1912, xxxii. 298.

(Salin and Reilly,[20] Mery, Salin, and Wilborts,[21] Menetrier and Avezou,[22] and Hallé[23]). In these cases two or three injections of ordinary antimeningococci serum were said to have been without effect, while the injection of the antiparameningococcus serum was followed by prompt improvement. Hallé, noting that only extracellular cocci were present in the cerebrospinal fluid after the ordinary serum had been given, did not wait for cultures of the diplococcus before resorting to the antiparameningococcus serum. He believes that a mixture of para and true meningococcus serum will give good results in the treatment of meningitis, though he agrees with Netter[24] that polyvalent serum is best. Netter uses a mixture of the two sera, but believes that a polyvalent serum, like that made in America, and which has given him excellent results, fulfills all requirements.

SUMMARY AND CONCLUSIONS.

The parameningococci of Dopter are culturally indistinguishable from true or normal meningococci, but serologically they exhibit differences as regards agglutination, opsonization, and complement deviation.

Because of the variations and irregularities of serum reactions existing among otherwise normal strains of meningococci it does not seem either possible or desirable to separate the parameningococci into a strictly definite class. It appears desirable to consider them as constituting a special strain among meningococci not, however, wholly consistent in itself.

The distinctions in serum reactions between normal and para meningococci are supported by the differences in protective effects of the monovalent immune sera upon infection in guinea pigs and monkeys.

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[20] Salin, H., and Reilly, J., *Bull. et mém. Soc. méd. d. hôp. de Paris,* 1913, xxxv, series 3, 423.

[21] Mery, H., Salin, H., and Wilborts, A., *Bull. et mém. Soc. méd. d hôp. de Paris,* 1913, xxxv, series 3, 411.

[22] Menetrier, P., and Avezou, J., *Bull. et mém. Soc. méd. d. hôp. de Paris,* 1914, xxxvii, series 3, 45.

[23] Hallé, *Bull. et mém. Soc. méd. d. hôp. de Paris,* 1914, xxxvii, series 3, 149.

[24] Netter, *Bull. et mém. Soc. méd. d. hôp. de Paris,* 1914, xxxvii, series 3. 53.

It is therefore concluded that it is highly desirable to employ strains of parameningococcus in the preparation of the usual polyvalent antimeningococcic serum. It remains to be determined whether it is better to employ the parameningococci along with normal meningococci in immunizing horses, or to employ normal and para strains separately in the immunization process and to combine afterwards, in certain proportions, the sera from the two kinds of immunized horses.

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A METHOD FOR ESTIMATING THE BACTERIA IN THE CIRCULATING BLOOD IN RABBITS.*

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Not a few of the determinations in experimental bacteriology have to do with the testing of bacteria for their infectious power. The laboratory animals principally employed for this purpose are small, and afford few reliable criteria which can be used to decide the result. The criterion which, because of its very definite character, is usually relied upon is the development of severe illness and the intervention of death; but this criterion cannot take account of degrees of intensity of infection, except as they affect the period of survival of the animal after inoculation, which is manifestly a coarse differential. All who have conducted so called virulence tests with bacteria have felt the need of other means of differentiation than mere survival or death of the inoculated animal. In the course of some experiments on the therapeutics of streptococcal and pneumococcal infections, this need became imperative, so that a means was sought that should yield data capable of comparison at different periods and intervals after inoculation, and which did not rely alone on the ultimate result of recovery or death.

The starting point of the quest was the well known phenomenon, first observed by von Fodor[1] and investigated accurately by Wyssokowitsch,[2] namely, that bacteria injected into the circulation are soon filtered out of the blood and, according to their degree of infectiousness, are destroyed or reappear, multiply, and, when of sufficient power, ultimately cause death. Hence the question which arose was whether the disappearance, reappearance, and multiplication proceed with a degree of regularity and consistency rendering a numerical estimate feasible of the bacteria in the circulating blood.

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* Received for publication, June 28, 1914.

[1] von Fodor, J., *Arch. f. Hyg.*, 1886, iv, 130.

[2] Wyssokowitsch, W., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1886. i. 3.

The later studies of Weil[3] and of Reichstein,[4] which deal with the fate and the estimation of streptococci within the blood stream, offered an encouraging affirmative on this point, so that the next question to arise was whether blood taken repeatedly from superficial vessels sufficed for the purposes of the estimation.

EXPERIMENTAL PART.

Bacteria and Injections.—The streptococcus used in the following experiments was originally isolated from the throat of a scarlet fever patient. It is a typical hemolytic, Gram-positive, chain-forming streptococcus. It has been repeatedly passed through rabbits and, at the beginning of this work, 0.25 of a cubic centimeter of a twenty-four-hour bouillon culture[5] per kilo of body-weight killed rabbits within one to five days. The pneumococcus used belongs to group I according to Neufeld's classification. Its virulence has been maintained by repeated passages through mice and rabbits. 0.001 of a cubic centimeter of a twenty-four-hour bouillon culture per kilo of body-weight kills rabbits within one to three days. In all experiments the bacteria were injected into the ear veins.

Taking the Blood.—The uninjected ear was carefully shaved and washed with 95 per cent. alcohol. The marginal vein was pricked and a few drops of blood allowed to flow out before any was taken for the cultures. Then a definite number of drops were caught directly into sterile Petri dishes. A tube of agar, previously melted and cooled to 42° C., was immediately poured into the dishes and the blood and agar were thoroughly mixed. Two or more plates were made from each rabbit, the amount of blood varying from one to ten drops, according to the supposed degree of infection. In this way plates were obtained on which the colonies could be easily counted and the various plates compared.

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[3] Weil, E., *Ztschr. f. Hyg. u. Infectionskrankh.*, 1911, lxviii, 346.

[4] Reichstein, S., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1913, lxxiii, 209.

[5] We have found blood bouillon (3 to 5 drops of sterile defibrinated rabbit blood in a tube of 5 c.c. of beef infusion bouillon) to be an excellent medium for streptococci and pneumococci. The difficulty of "infecting" large quantities of medium with loop inoculations from bouillon to bouillon is eliminated (Gillespie). The bacteria do not lose this virulence as readily in this medium as in ordinary bouillon. Another advantage is that the bacteria live in this medium for long periods of time without being transferred. Streptococci will live for 6 months when the cultures are kept in the dark at low temperature; it is not necessary to seal the tubes in any way. Pneumococci will live for at least 6 weeks. This medium was used throughout the experiments instead of plain bouillon.

The above procedure permits of frequent taking of blood with the least possible disturbance to the rabbit. Contaminations rarely occur if the ear is properly cleansed and a few drops are allowed to flow out before taking the blood for the cultures. Blood from the first few drops adheres to the surface of the ear and the following drops run over this without coming in contact with the skin. The accuracy of the method might be objected to, because the measuring of the blood is as inaccurate as the size of one drop of blood may differ from another. If the veins are pricked by a stab with the same sharp-pointed instrument and the surface of the ear is held in a perpendicular position so as not to allow the blood to collect in large quantities before flowing off, drops of fairly constant size can be obtained and the number of colonies on various plates of the same number of drops is remarkably uniform. The accuracy of the procedure can also be tested by infecting a series of rabbits with the same quantity of a bacterial suspension per kilo of body-weight and taking cultures at stated intervals in a short time after the injections. Experiment 1, as given below, was done with this object in view.

STREPTOCOCCUS INFECTIONS.

Experiment 1.—Each of seven rabbits was injected intravenously with 0.25 c.c. of a fresh bouillon culture of streptococci per kilo of body-weight. Cultures were made from the opposite ear 30 minutes and 5½ hours afterwards. In this, as in all other experiments, the number of colonies was calculated on the basis of ten drops of blood. The results are given in table I.

TABLE I.

	Rabbit 1.	Rabbit 2.	Rabbit 3.	Rabbit 4.	Rabbit 5.	Rabbit 6.	Rabbit 7.
Colonies at 30 min..........	140	145	135	100	130	130	100
Colonies at 5½ hrs.........	8	9	20	22	10	12	10

Many experiments of this character were performed and similar results were always obtained. The first cultures were made after ample time had elapsed for a thorough and uniform distribution of the bacteria in the blood stream and the number of colonies obtained from the different rabbits shows only slight variations. At the next bleeding, five hours later, the plates were still uniform, although a great reduction in the number of colonies had occurred. These results are probably due to the fact that this initial disappearance of the bacteria from the blood depends largely upon mechanical forces which are about the same in each animal. A biological reaction between the host and the infecting organism had evidently played a very slight rôle up to this time; otherwise, such uniform results could not be expected. Such an experiment also shows that this method of taking blood gives dependable data.

In following the progress of the infections further, it was found that they may take one of three courses, mainly depending upon the virulence of the bacteria. A series of experiments was carried out just after the bacteria had been passed through a number of rabbits. The infections ran an acute course, the blood was never free of bacteria, and the autopsies showed no evidence of localization. After two months' cultivation on artificial media, no animal passages being made, the same amount of bacteria caused a chronic infection in a majority of the animals. There were longer or shorter intervals during which the septicemias were slight or entirely absent. After a time the bacteria suddenly reappeared in the blood and the animals died within a few hours to two days. Autopsies showed localized infections, from which the second blood invasion undoubtedly came. When the bacteria were still less virulent, they permanently disappeared from the blood and the animals recovered. Examples of these types of infection are reported in the following tables.

Experiment 2.—Each rabbit was given 0.25 c.c. per kilo of body-weight of the same suspension of streptococci into the ear vein. The blood for the cultures was taken from the opposite ear at the times indicated in the protocols. The number of colonies was estimated on the basis of ten drops of blood.

An examination of table II shows that the infections, with the exception of rabbits 2 and 3, ran a remarkably uniform course. From the time of the injections to about five hours afterwards there is a rapid decrease in the number of bacteria. From five to six

TABLE II.[6]

Animal.	Time of bleeding and number of colonies.											
Rabbit 1,	30 min.	2 hrs.	6 hrs.	10 hrs.	22 hrs.	48 hrs.	72 hrs.	Died 1 hr. after the last bleeding.				
weight 1,800 gm.	52	1	18	12	9	100	×					
Rabbit 2,	30 min.	2 hrs.	6 hrs.	11 hrs.	23 hrs.	30 hrs.	47 hrs.	56 hrs.	Was dead at 64 hrs.			
weight 2,000 gm.	200	5	100	200	1000	×	×	950				
Rabbit 3,	30 min.	2 hrs.	6 hrs.	10 hrs.	22 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	126 hrs.	138 hrs.	Dead at 144 hrs.
weight 1,600 gm.	110	9	13	35	65	1500	×	100	75	×	100	
Rabbit 4,	30 min.	6 hrs.	18 hrs.	Dead at 36 hrs.								
weight 1,500 gm.	25	100	2000									
Rabbit 5,	30 min.	2 hrs.	6 hrs.	11 hrs.	23 hrs.	30 hrs.	36 hrs.	Dead at 38 hrs.				
weight 1,750 gm.	75	5	70	245	×	×	×					
Rabbit 6,	30 min.	3 hrs.	18 hrs.	24 hrs.	42 hrs.	48 hrs.	Dead at 50 hrs.					
weight 1,100 gm.	50	6	1000	2000	×	×						

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[6] In the tables, × indicates that the plates were so heavy that the colonies could not be counted.

hours afterwards the bacteria begin to increase in number and septicemia becomes heavier and heavier until the death of the animal. The rabbits died in from one and a half to three days. Rabbits 2 and 3, especially 3, showed some variations from this rapid, progressive course. Rabbit 3 lived six days and its blood cultures give a possible explanation for this. At seventy-two hours the blood had reached so great a septicemic state that the animal should have succumbed on the following day. But the rabbit lived and the blood culture showed a retrogression of the infection for two days. This was followed by another exacerbation which was also overcome and succeeded by another retrogression. The rabbit died on the sixth day with a low culture; but six hours elapsed between the last bleeding and the death of the animal. This gave sufficient time for another ascension, which probably occurred, and as a result of which the rabbit died. Such fluctuations or crises·cannot be ascribed to the technique, since they were often observed, and also a temporary reduction in the number of bacteria was always accompanied by a prolongation of the rabbit's life. Infections running such zigzag courses are positive indications that a two-sided warfare occurs within the body of the host; the bacteria make advances which are successfully met by the defensive power of the rabbit, but, not being entirely killed out, they make another trial. Such a struggle may be prolonged for several days, and the final results depend upon the relative strengths of the aggressive forces of the invading bacteria and the defensive resources of the infected animal.

Experiment 3.—This experiment was carried out two months after experiment 1. The same strain of streptococcus was used in this experiment and the same quantities of bouillon culture were injected. This strain had been kept on artificial media continuously and had evidently suffered a loss of virulence. The technique was the same as in experiment 1. Two typical rabbits are reported in table III.

The courses of the infections reported in table III show that the bacteria had lost in aggressiveness but they still possessed an insusceptibility to the destructive influences of the host and were yet able to carry on a local fight in some more or less inaccessible place. The local lesions probably lowered the vitality of the animals to

TABLE III.

Animal.	Time of bleeding and number of colonies.											
Rabbit 1,	30 min.	2 hrs.	6 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	140 hrs.	144 hrs.	168 hrs.	192 hrs.,
weight 1,540 gm.	76	0	0	0	0	0	0	0	0	0	150	rabbit died.
Autopsy.	Pleurisy with effusion. Streptococci abundant in the fluid. Smear from the heart's blood heavily positive.											
Rabbit 2,	30 min.	5 hrs.	12 hrs.	24 hrs.	36 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	144 hrs.	168 hrs.	192 hrs.
weight 1,720 gm.	30	1	80	50	30	30	25	0	10	50	200	×
Autopsy.	216 hrs., rabbit died. Pericarditis, and extensive cellulitis over the abdomen. Smears from these lesions were heavily loaded with streptococci.											

such an extent that the bacteria were able to produce a general infection. Or, on the other hand, the life within the tissues of the host increased the infectivity of the bacteria and they were able to overcome the opposition offered by the rabbit.

Experiment 4.—This experiment was made one month after experiment 3. The bacteria had been kept on artificial media during this time. The quantities of culture used and other points of technique were the same as in the preceding experiments. Three rabbits are given in table IV to represent the nature of infections obtained at this time.

In experiment 4 the bacteria were almost devoid of infecting power and they behaved very much as saprophytes; they rapidly disappeared from the circulation and were not able to reappear. The rabbits showed no signs of disease and remained in perfect condition.

PNEUMOCOCCUS INFECTIONS.

A number of experiments were carried out with the pneumococcus similar to those just described with the streptococcus. Depending upon the virulence and the number of bacteria injected, a pneumococcic infection in the rabbit may take any one of the three courses described in the streptococcic infections. The initial decrease in the number of bacteria in the blood may not be as complete as with the streptococcus, especially if a very large number of bacteria are injected. Pneumococci usually begin to reappear in the blood earlier than streptococci. The infections are more acute and severe. The types of infection are even more defined than with the streptococcus. The acute infections last from two to three days. In from two to three hours the bacteria have largely disappeared from the blood. Cultures taken at five hours usually show an increase in the number of colonies and this reaches a heavy septicemia in from eighteen to twenty-four hours. The animals die within forty-eight to seventy-two hours. Rabbit 1 in table V is an example of this type of infection. If the bacteria have lost in virulence or if a smaller number is injected, a chronic infection occurs. Rabbit 2 of table V represents this class typically. The bacteria disappear from the blood more rapidly and the reappearance is delayed and the increase is slight and is followed by a second decrease. The rabbits usually have a low septicemia for several days. This is followed by a rise and the animal dies within one to two

TABLE IV.

Animal.	Time of bleeding and number of colonies.							
Rabbit 1, weight 1,380 gm.	30 min. 75	2 hrs. 4	20 hrs. 3	26 hrs. 5	44 hrs. 20	66 hrs. 0	72 hrs. 0	The bacteria never reappeared in the blood and the rabbit was in perfect condition 1 mo. later.
Rabbit 2, weight 1,620 gm.	10 min. 180	4 hrs. 0	20 hrs. 0					The blood remained sterile and the rabbit was in good condition after 1 mo.
Rabbit 3, weight 1,200 gm.	3 hrs. 7	20 hrs. 0	44 hrs. 12	64 hrs. 0	112 hrs. 0			Bacteria never reappeared in the blood, and the rabbit continued well.

days. At autopsy severe local lesions are always found. The localization may be in the pericardium, pleura, peritoneum, or in the subcutaneous tissues of the abdomen. In one rabbit localization occurred in the kidneys. Any two or more of these places may be involved in the same animal, but many times only one is affected. The bacteria accumulate in great quantities in these localities; when present, septicemia almost invariably precedes death. In the third type the rabbit masters the situation within a few hours; the bacteria disappear from the blood permanently and the animal continues in perfect condition. Rabbit 3 in table V falls in this class.

SUMMARY.

When rabbits are injected intravenously with a quantity of virulent streptococci or pneumococci sufficient to cause death within two to four days the septicemia takes a definite course with slight variations. The bacteria rapidly decrease in number from the time of the injection to from two to four hours, at which time the blood is sterile or contains only a few bacteria. Within five to six hours the bacteria reappear in the blood and steadily increase until the death of the animal. If the bacteria are less virulent, the same quantity of culture causes a chronic type of infection. The same initial decrease in the number of bacteria occurs. The reëntrance into the blood is somewhat delayed, the septicemia does not reach the height obtained in the acute cases, and a second fall occurs within the course of a few hours. These rabbits show a low blood invasion or a sterile blood culture for several days. During this time they become emaciated to a marked degree. Then the low septicemia rapidly rises or the rabbit with a sterile culture develops a severe septicemia within a few hours and death takes place from a few hours to two days thereafter. In this type of infection local lesions, pericarditis, pleurisy, peritonitis, etc., are usually found. In the infections which run an acute course no gross lesions are found. If the bacteria are still less virulent they never reënter the blood after the initial disappearance and the rabbits remain in good condition. In order to obtain uniform results, the quantity of bacteria injected must not be so large that the bacterial substances

TABLE V.

Animal.	Time of bleeding and number of colonies.										
Rabbit 1,	30 min.	3½ hrs.	22 hrs.	27 hrs.	In 44 hrs. rabbit was found dead.						
weight 1,450 gm.	150	0	×	×							
Rabbit 2,	5 min.	30 min.	1½ hrs.	3 hrs.	4½ hrs.	7 hrs.	11 hrs.	21 hrs.	25 hrs.	28 hrs.	48 hrs.
weight 1,700 gm.	350	3	0	0	3	15	1	100	350	200	125
	72 hrs.	96 hrs.	Dead 120 hrs. Autopsy showed pleurisy, pericarditis, and cellulitis over the abdomen. Films from these places were heavily loaded with pneumococci.								
	100	200									
Rabbit 3,	10 min.	4 hrs.	20 hrs.	The blood remained sterile and the rabbit fully recovered.							
weight 1,620 gm.	180	0	0								

carried in are sufficient to cause an intoxication of the animal. If the quantity of bacteria injected is below this point the course of the infection depends largely upon the virulence of the infecting organisms. Yet variations in the natural resistance of individual animals may be sufficient to cause quite marked irregularities in the course of the infection. Pneumococci can be standardized so as to produce a particular type of infection more easily than streptococci. In general infections such as those produced by streptococci and pneumococci the number of the bacteria present in the circulating blood at a given time supplies accurate and delicate information regarding the severity of the disease. When the object is to determine the degree of virulence of bacteria, or of the efficiency of an experimental therapeutic method, the mere physical condition and mere death of the inoculated animals are not sufficient and satisfactory guides to the desired information. The death of the inoculated animal and the recovery of the infecting bacteria at autopsy do not give complete information concerning the intensity and course of the infection occurring during life. A large number of bacteria found in the blood and tissues at autopsy do not necessarily prove the existence of a heavy infection before the onset of the death agony, since it is a well known fact that bacteria multiply with enormous rapidity, once the natural resistance of the animal has been overcome. Therefore, if merely the life and death of the animal and autopsy findings must serve as our only guides, we shall lose much incidental information, perhaps of fundamental value. This may be especially true as regards the search for curative substances. Again, the individual animals of the same species, age, and apparently of identical physical condition react to the aggressive force of the infecting organisms variously. This fact is readily found out by the injection of a series of rabbits with lethal quantities of bacteria per body-weight, and by making tests at various periods before death results, which, in the case of streptococci, ranges from one to six days. Consequently a method which enables the determination of the degree and progress of the infection at any desired period is of obvious advantage.

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LOCALIZATION OF THE VIRUS AND PATHOGENESIS OF EPIDEMIC POLIOMYELITIS.*

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Since epidemic poliomyelitis is an affection chiefly of the central nervous tissues, it may be supposed that the nervous organs possess a special affinity for its microbic cause.[1] Once the virus of the disease has gained access to, and multiplied within, the nervous tissues, it survives there, apparently, longer than it does in other organs in which, under ordinary conditions, it occurs far less regularly. It has been shown by experiment that the poliomyelitic virus readily reaches the central nervous system when it is brought into relation with the peripheral nerves. It is in this manner that infection is induced when the virus is brought into contact with the nasal mucosa, sciatic nerves, and probably also when it is injected into the subcutaneous tissues and peritoneal cavity.[2] Thus deposited, the virus ascends by way of the nerves to the olfactory lobes of the brain or to the spinal cord and intervertebral ganglia. It is to be assumed that in the case of the spinal nerves the ascent is by way of the afferent or sensory fibers; in the case of the nasal membrane, along the olfactory fibers. Hence the virus is carried both by nerves of common and of special sensation. In these instances the virus is brought into relation, not with special end organs, but with nerve fibrils, along which it travels. It appears, however, that the virus may enter the nerves by way of specialized end organs, which are themselves not appreciably injured by it.

INTRAOCULAR INOCULATION.

The demonstration that the virus of poliomyelitis may penetrate the uninjured sensory end organs has been made in connection with the optic nerve. An emulsion of the spinal cord carrying the active

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[1] Flexner, S., *Jour. Am. Med. Assn.*, 1910, lv, 1105.

[2] Flexner, S., and Amoss, H. L., *Jour. Exper. Med.*, 1914, xix, 411.

virus and free from bacterial contamination can be injected into the vitreous chamber of the eye without causing appreciable inflammation. A small quantity of the vitreous humor is aspirated, under ether anesthesia, by means of a tuberculin syringe carrying a very fine needle, and replaced by an emulsion of the virus. The cloudiness thus caused tends to disappear, and the vitreous body to return to normal. After a variable incubation period, symptoms of experimental poliomyelitis appear, paralysis develops, and the pathological findings are characteristic of the disease.

Experiment 1.—Macacus rhesus. May 29. Withdrew under ether anesthesia as stated 0.1 c.c. vitreous humor by puncture of sclera and introduced 0.2 c.c. emulsion of active virus. June 7. Excitable. June 9. Weakness of arms. June 10. Ataxic. June 12. Paralysis of right arm; weakness of left leg; both eyes normal. June 15. Arms and legs weak. From this date the condition remained stationary until June 22, when death occurred from diarrhea.

The autopsy revealed old and recent dysenteric lesions of the large intestine. No visible changes were observed in the spinal cord or eyes. However, microscopical examination of the spinal cord, medulla, and intervertebral ganglia shows typical lesions of poliomyelitis. The retina of the inoculated eye appears normal.

Experiment 2.—Macacus rhesus. June 12. Withdrew, in the manner of experiment 1, 0.1 c.c. vitreous humor from the left eye and injected 0.2 c.c. paper filtrate of an emulsion of virus. The cloudiness of the vitreous humor did not entirely clear up. June 19. Excitable. June 22. Tremor of head; weakness of left leg; ataxia. June 23. Paralysis of left arm; weakness of right arm; double ptosis and slight left facial paralysis; weakness of left leg. June 24. Prostrate; etherized.

The spinal cord, medulla, and intervertebral ganglia present typical lesions of poliomyelitis. The ophthalmic ganglion on the inoculated side shows also a diffuse cellular infiltration.

The two experiments given confirm the supposed affinity which the poliomyelitic virus possesses for nervous tissues and they indicate also that the virus is capable of penetrating highly specialized end organs in order to reach peripheral nerves, along which it penetrates to the central nervous system. Incidentally it shows that the virus may pass from peripheral nerves into the adjacent ophthalmic ganglion, but whether directly from the eye, or indirectly after infection of the central nervous organs as occurs with the Gasserian ganglia, does not appear.[8]

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[8] Landsteiner, K., and Levaditi, C. (*Compt. rend. Soc. de biol.*, 1909, lxvii, 787) succeeded in one instance in producing paralysis by inoculating the virus into the anterior chamber of the eye.

DISTRIBUTION OF THE VIRUS AFTER INTRAVENOUS INOCULATION.

It is established that experimental poliomyelitis may be caused with more or less regularity by insuring that the virus reaches the central nervous organs by way of the peripheral nerves. When the virus is brought directly into relation with the central nervous system by intracerebral and intraspinous injections the most constant results are secured. Probably the less constant effects which follow injection of the virus into the peripheral nerves result from the fact that the greater the distance the virus is compelled to travel along nerves, the more chances there are for miscarriage of infection, either by reason of too great dilution, or failure of the virus to reach the central organs at all.

In all these instances the affinity of the nervous organs for the virus may be exerted directly, since the virus is brought either immediately into relation with the nervous tissues, or reaches them directly through lymphatic communication. When the virus is brought to the nervous organs by means of the blood, it is at first separated from the tissues themselves by the vessels and other structures interposed between the blood itself and the nervous tissue. For this reason it has been observed that, while small or even infinitesimal doses of the virus suffice to induce quite constant infection by the intranervous mode of inoculation, large quantities of the virus produce only occasional and inconstant infection, when injected directly into the blood.

The cause of this discrepancy has already been traced to an apparent inability of the virus to enter directly the substance of the brain and spinal cord from the blood.[4] In order to reach these organs, the virus must, it appears, leave the blood and pass into the cerebrospinal fluid, with which it reaches the interstices of the tissues. Since the cerebrospinal liquid is a product of the activity of the choroid plexus, it has been assumed that the virus must first penetrate that structure. Experiments have been performed in order to study this phase of the subject more closely.

Infectivity of Organs.—When the virus is injected directly into the blood it is quickly distributed throughout the circulatory system in the manner in which other microörganisms are distributed. In due time it may be assumed that the virus is deposited in certain organs, since experiment has shown that it does not remain long in the circulating blood.[5] Hence it is readily possible to ascertain the distribution of the virus by sacrificing at intervals the infected animals, and inoculating emulsions of the organs themselves. In this manner it can be determined whether the virus is distributed mechanically, or according to the affinity which the several organs display toward it.

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[4] Flexner and Amoss, *loc. cit.*

The next experiments to be described have been devised to answer this question.

Experiment 3.—Macacus rhesus. May 4. 250 c.c. of a centrifugalized suspension of the spinal cord and medulla containing the active virus were injected into one of the superficial veins of the leg. Three days later, on May 7, the animal was etherized and the spinal cord, medulla, crura cerebri, and intervertebral ganglia were removed aseptically. These were made into 5 per cent. suspensions which were injected intracerebrally into three *Macacus rhesus* monkeys, A, B, and C.

Monkey A.—May 7. 2 c.c. of emulsion of intervertebral ganglia injected. May 10. Excitable. May 12. Right arm weak; left arm paralyzed. May 13. Prostrate; etherized. Typical poliomyelitis.

Monkey B.—May 7. Injected 2 c.c. of emulsion of spinal cord and medulla. This animal developed no symptoms and remained well indefinitely.

Monkey C.—May 7. Injected 2 c.c. of emulsion of crura cerebri. No symptoms of poliomyelitis developed and the animal remained well indefinitely.

Experiment 4.—Macacus rhesus. Apr. 16. 240 c.c. of centrifugalized virus were injected intravenously. Four days later, Apr. 20, the animal was etherized. There were removed aseptically for inoculation: spleen, bone marrow, kidneys, spinal cord and medulla, which were made into 5 per cent. emulsions and injected intracerebrally into *Macacus rhesus* monkeys, D, E, F, and G.

Monkey D.—Apr. 21. Received 2 c.c. of the suspension made from portions of spinal cord and medulla. No symptoms of poliomyelitis developed, and the animal remained normal.

Monkey E.—Apr. 21. Received 2 c.c. of a suspension of portions of each kidney. No symptoms of poliomyelitis developed, and the animal remained normal.

Monkey F.—Apr. 21. Received 2 c.c. of a suspension of the spleen. Apr. 21. Excitable; ataxic. Apr. 25. Both legs and right arm paralyzed. Apr. 27. Died. Typical poliomyelitis.

Monkey G.—Apr. 21. Received 2 c.c. of suspension of bone marrow of both femurs. Apr. 24. Excitable. Apr. 29. Tremor; weakness of neck. Apr. 30. Prostrate; etherized. Typical poliomyelitis.

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[5] Clark, P. F., Fraser, F. R., and Amoss, H. L., *Jour. Exper. Med.*, 1914, xix. 223.

Experiment 5.—Macacus rhesus. Mar. 31. Intravenous injection of 250 c.c. of centrifugalized virus. Five days later, Apr. 5, etherized and portion of spleen, bone marrow, spinal cord and medulla were removed aseptically and made into 5 per cent. emulsions and injected intracerebrally into *Macacus rhesus* monkeys, H, I, and J.

Monkey H.—Apr. 5. Injected 2 c.c. of suspension of spinal cord and medulla. No symptoms developed and the animal remained normal.

Monkey I.—Apr. 5. Injected 3 c.c. of the suspension of the spleen. Apr. 8. Excitable. Apr. 11. Ataxic. Apr. 12. Tremor; prostrate. Apr. 13. Died. Typical poliomyelitis.

Monkey J.—Apr. 5. Injected 3 c.c. of suspension of bone marrow from both femurs. Apr. 10. Excitable; ataxic; weakness of left leg. Apr. 17. Legs and left arm paralyzed; prostrate; etherized. Typical poliomyelitis.

The sections of the central nervous organs show not only marked lesions of poliomyelitis, but those of the fourth ventricle reveal an infiltration of the choroid plexus with mononuclear cells.

This series of experiments is consistent in exhibiting that, in spite of the essential affinity which the spinal cord and brain exhibit for the poliomyelitic virus, they are, nevertheless, unable to remove it directly from the blood, prior to some change taking place in the structures (vascular or secretory) that preside over the production of the cerebrospinal fluid; while the spleen and bone marrow, but not the kidney, readily remove it from this source. The fact that the intervertebral ganglia are capable of readily removing part of the virus from the blood shows, first that their relation to the blood vessels differs from that of the brain and spinal cord, and second explains the constant and early involvement of these structures in the poliomyelitic process. The ganglia, therefore, appear capable of obtaining the virus from two sources, namely, directly from the blood, and indirectly from the cerebrospinal fluid.

Moreover, the poliomyelitic virus may be retained alive in the body for a considerable period of time without gaining access to the interior of the central nervous organs.

Experiment 6.—Macacus rhesus. Apr. 18. Injected intravenously 250 c.c. of centrifugalized virus. No symptoms of poliomyelitis developed and seventeen days later, May 5, the animal was etherized. 5 per cent. suspensions of spleen and central nervous tissues were prepared and inoculated intracerebrally in monkeys K and L. Microscopic examination of sections of the spinal cord, medulla, and intervertebral ganglia prove them to be free from lesions of poliomyelitis.

Monkey K.—May 6. 2 c.c. of emulsion of medulla and spinal cord injected. No symptoms developed, and the animal remained normal.

Monkey L.—May 6. 2 c.c. of suspension of spleen injected. May 12. Excitable; ataxic. May 16. Arms weak; etherized. Poliomyelitis.

This experiment indicates, first that the virus of poliomyelitis is capable of surviving for a considerable period in the interior of the body, without inducing an infection of the central nervous system; and next that this long sojourn is not without effect on the quality of the virus, which would appear to have been weakened by the action of the spleen.

Effect of Aseptic Meningitis.—The permeability of the meninges for the contents of the blood is increased by inflammation of those structures. The introduction of sterile alien blood serum into the subarachnoid spaces causes an aseptic inflammation of mild degree that reaches its maximum in twenty-four hours, and then subsides. The inflammation is marked by emigration into the pia-arachnoid, cerebral ventricles, and choroid plexus of polymorphonuclear leucocytes chiefly, and by the escape of plasma. Neither the ependymal epithelium nor the perivascular lymphatics show appreciable change.

Experiments were conducted to ascertain the effect of this inflammation on the penetration of the virus of poliomyelitis into the central nervous organs.

Experiment 7.—Control. *Macacus rhesus.* Apr. 23. Injected intravenously 25 c.c. of centrifugalized suspension of spinal cord and medulla of paralyzed monkey. No symptoms developed and the animal remained normal.

Experiment 8.—Control. *Macacus rhesus.* Apr. 23. 50 c.c. of the centrifugalized suspension of virus used in the previous experiment were injected intravenously. The animal remained normal.

Experiment 9.—*Macacus rhesus.* Apr. 22. Injected intraspinously 3 c.c. of 40 per cent. inactivated horse serum. Apr. 23. Lumbar puncture; 0.5 c.c. of turbid fluid containing large numbers of white corpuscles was obtained. Injected intravenously 25 c.c. of centrifugalized suspension of virus as in experiments 7 and 8. Apr. 26. Excitable; legs weak. Apr. 30. Arms also weak; etherized.

Microscopic examination of the medulla, spinal cord, and intervertebral ganglia reveals pronounced lesions of poliomyelitis. While vascular lesions are everywhere pronounced, lesions of the nerve cells, interstitial substance, and meninges are also marked. The choroid plexus of the fourth and lateral ventricles is included in the sections. The plexus of the fourth ventricle shows definite infiltration with mononuclear (lymphoid) cells; the plexus of the lateral ventricle is less infiltrated. The blood vessels in the floor of the fourth ventricle are heavily infiltrated, while the deeper vessels are less affected, and those beneath the lateral ventricle are unaffected.

Experiment 10.—Macacus rhesus. May 5. Injected intraspinously 3 c.c. of 40 per cent. inactivated horse serum. May 6. Injected intravenously 25 c.c. of centrifugalized suspension of active virus. May 9. Excitable. May 13. Right arm paralyzed. May 14. Died.

The microscopic examination of sections of the medulla, spinal cord, and ganglia shows poliomyelitic lesions of moderate degree. The most pronounced lesions occur in the floor of the fourth ventricle. Vascular lesions are nowhere severe.

The foregoing observations raise anew the question as to the path traversed by the virus from the blood to the cerebrospinal fluid, and thence to the nervous tissue. Since the cerebrospinal liquid is the product of the secretory activity of the choroid plexus, it has been assumed that the impermeability of the plexus for most foreign products results in the exclusion also of microörganisms, as long as the secreting structures remain intact. Apparently such moderate quantities of the poliomyelitic virus as are contained in twenty-five to fifty cubic centimeters of a clear suspension of the spinal cord and medulla, taken from a paralyzed monkey, may be insufficient to inflict the necessary damage upon the choroid plexus, while still larger quantities may suffice to accomplish this. When, however, the superficial structures of the nervous organs, such as the meninges and choroid plexus, are put into a state of mild chemical inflammation, their permeability is increased, so that what was before an inadequate quantity of virus is now rendered sufficient to cause poliomyelitic infection.

Another fact has emerged from these experiments: while the blood vessels of the spinal cord and brain may show no unusual degree of pathological alterations, definite lesions of an infiltrative nature may appear in the choroid plexus itself. Before, however, a decision is reached as to the relation of the histological lesions to the escape of the virus from the blood, it is desirable to determine the nature of the lesions in animals that have developed paralysis from large unaided injections of the virus, administered intravenously.

Lesions Caused by Intravenous Injections.—While rhesus monkeys show almost no difference in susceptibility to the action of the poliomyelitic virus when introduced directly into the brain, they exhibit distinct differences when injections are made into the peripheral parts of the nervous system, or into the blood. Moreover, the quality of the virus itself is brought out by the site of inoculation, since a specimen that is of less than maximal activity may be infectious even in minute doses, when introduced into the brain, and either not active at all, or slightly infectious when injected into the blood, or even into the sciatic nerves. As the experiments that follow show, the quantity of the centrifugalized suspension carrying the virus required to cause infection by the unaided blood route usually exceeds fifty cubic centimeters, while even 200 or more sometimes fail, although a few tenths of a cubic centimeter of the same virus succeed when introduced into the brain.

Before describing the lesions present in the spinal cord, brain, and intervertebral ganglia, caused by intravenous injections, it is desirable to present briefly the nature of the lesions of experimental poliomyelitis such as result from other modes of inoculation. The several lesions may be considered as they affect first the meninges, second the spinal cord, third the medulla and pons, fourth the cerebrum, and fifth the ganglia.[6]

The meninges of the cord and medulla show, as a rule, mononuclear cellular infiltration most pronounced adjacent to, or surrounding, the blood vessels which enter the fissures of the cord and are present in the floor of the fourth ventricle. The general infiltration of the pia-arachnoid is interstitial and as a rule not heavy, while the invasion about the vessels within the perivascular lymphatics is usually heavy, and sometimes is nodular. The spinal cord presents lesions most pronounced in the anterior gray matter, less marked in the posterior gray matter, and least present in the white matter. They are perivascular, interstitial, and parenchymatous. The vascular lesions, which are often pronounced, extend inward from the meninges; the interstitial ones are associated with the presence of mononuclear, to a less extent of polynuclear cells, rarely of red corpuscles, and commonly of serum. Actual necrosis of the ground substance arises, but is uncommon on a large scale. The anterior gray matter is rarely wholly destroyed at certain levels. The interstitial lesions can, in some instances, be traced outwards, directly from affected vessels. The lesions of the parenchyma consist of degeneration and necrosis of ganglion cells, occurring chiefly but not exclusively in the anterior gray matter. The necrotic cells are commonly invaded by phagocytes, the so called neurophages. It is not usual for definite relation to be obvious between the altered blood vessels and the affected interstitial substance or parenchyma. The lesions of the medulla resemble those of the spinal cord, except as they are modified by differences in structure. The vessels most infiltrated are those present immediately beneath the fourth ventricle; the deeper lying vessels tend to be less affected, and the very small branches throughout the part are involved inconstantly. The focal interstitial lesions tend to be smaller than those of the cord. No definite relation can, as a rule, be made out between the vascular and interstitial changes. Because of the smaller size and less uniform distribution of nerve cells, the parenchymatous lesions are less conspicuous; they are, however, essentially identical with those of the cord. Lesions similar to those in the medulla occur in the pons and crura cerebri, but less frequently. The cerebrum is affected far less constantly than other parts of the nervous system. When present, the lesions are perivascular and focal interstitial. The cerebral meninges, as a rule, escape affection. The choroid plexus of the lateral and fourth ventricles has not been studied in all instances. When the virus has been introduced into the brain, cerebrospinal fluid, or nerves, the rule appears to be that the plexus escapes. However, exceptions to this rule occur, in which case lesions similar to those to be described as occurring after intravenous injection of the virus may arise (compare experiment 5, monkey J). The intervertebral ganglia are invariably affected. The lesions are of two main kinds, interstitial and parenchymatous, and are always focal. The cellular invasion proceeds from two sources; the pial investment and the blood vessels. In the former, direct extension may take place from the spinal meninges, or extension may occur by way of the connective tissue of the nerve roots. In the latter, extension seems to proceed from the blood vessels. It remains, however, to state that the involvement of the blood vessels may not arise through the general blood, but through inclusion of the vessels in the infiltrative process within the septa of the nerve roots. In rare instances the blood vessels present, as compared with other parts of the ganglia, an unusual degree of surrounding infiltration. The ganglionic nerve cells are destroyed in two ways: first, they are obliterated by focal accretions of mononuclear cells, and, second, by necrosis and neurophagocytosis, in the same manner as in the corresponding condition in the spinal cord.

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[6] Flexner, S., and Lewis, P. A., *Jour. Exper. Med.*, 1910, xii, 227.

With this description before us, we may now proceed to describe the lesions which arise as a result of infection by the intravenous mode of inoculation.

Experiment 11.—Macacus rhesus. Nov. 14. 100 c.c. of a Berkefeld filtrate of the virus were injected into the right saphenous vein. Nov. 24. Excitable; paralysis of the left leg and left side of the face. Nov. 25. Paralysis of arms and legs; weakness of back. Nov. 26. Prostrate. Dec. 1. Died.

The spinal cord, medulla, and ganglia show pronounced lesions. Those of the spinal cord affect the blood vessels, interstitial tissue, and nerve cells. They are not distinctive, and do not differ from the usual lesions. This fact is also true of the lesions in the medulla, which are most pronounced in the blood vessels in the floor of the fourth ventricle, and of the ganglia, where they are wide-spread.

Experiment 12.—Macacus rhesus. Nov. 7. Injected intravenously 54 c.c. of a Berkefeld filtrate of the virus. At the same time, under ether anesthesia, 5 c.c. of salt solution were introduced into the left cerebral hemisphere. Nov. 16. Excitable; ataxic. Nov. 17. Arms paralyzed; back weak. Nov. 18. Prostrate. Nov. 19. Etherized.

The lesions of the spinal cord, medulla, ganglia, and cerebrum are pronounced. Not only are the usual lesions present, but in addition severe affection of the blood vessels in the cord, medulla, and cerebrum occurs, from which infiltrations extend into the substance of the nervous tissues. The cerebral meninges at the site of the injection of salt solution also show infiltration.

Experiment 13.—Macacus rhesus. Jan. 30. 250 c.c. of centrifugalized suspension injected intravenously. Feb. 14. Excitable. Feb. 16. Weakness of legs. Feb. 17. Paralysis of arms. Feb. 18. Weakness of back. Feb. 19. Died.

The lesions are typical of poliomyelitis. The meninges of the spinal cord are diffusely infiltrated. The vascular lesions are moderate. There is widespread degeneration of the nerve cells, but none of the lesions are distinctive, or differ from those usually occurring.

Experiment 14.—Macacus rhesus. Oct. 30. Intravenous injection of 180 c.c. of centrifugalized virus. Nov. 9. The animal shows weakness and disturbance of vision. During the night it died.

The medulla, spinal cord, and ganglia are the seat of marked lesions of poliomyelitis. The vascular infiltration is heavy but the usual larger vessels only are affected. The cerebrum is devoid of lesions, while the crura cerebri are the seat of interstitial, but not of striking vascular lesions.

Experiment 15.—Macacus rhesus. Feb. 28. Intravenous injection of 250 c.c. of centrifugalized virus. Mar. 4. Excitable. Mar. 5. Arms and legs weak; ataxic. Mar. 6. Died.

The lesions of the spinal cord are perivascular, interstitial, and meningeal; of the ganglia, perivascular, with extension into the nerve roots. The medulla shows a high degree of affection of the blood vessels. The cerebrum has escaped, but the choroid plexus of the lateral ventricle, but not of the fourth ventricle, shows edema and perivascular cellular infiltration. The ependymal cells appear normal.

Experiment 16.—Macacus rhesus. Apr. 16. Intravenous injection of 250 c.c. of centrifugalized virus. During the injection a needle was kept in the lumbar spinal canal. Apr. 21. In the morning the arms and back were paralyzed. In the afternoon death occurred.

The lesions in this instance are very pronounced. The blood vessels within the spinal cord, medulla, and pons show wide involvement, while the interstitial tissue and nerve cells are affected only moderately. The cerebrum is devoid of lesions, while the choroid plexus of the lateral ventricles contains a slight accumulation of mononuclear cells about the blood vessels. The plexus of the fourth ventricle appears normal.

Experiment 17.—Macacus rhesus. Apr. 16. 240 c.c. of centrifugalized virus injected intravenously. Apr. 20. Lumbar puncture yielded a fluid containing an excess of white corpuscles. Apr. 21. Excitable; no paralysis; etherized.

The spinal cord, medulla, and pons show early vascular, but no other lesions. The ganglia, however, contain focal cellular infiltrations of small size, and a small number of single necrotic nerve cells. No changes were detected in the choroid plexus.

Experiment 18.—Macacus rhesus. Apr. 16. 240 c.c. of centrifugalized virus injected intravenously. Apr. 20. No symptoms appeared; etherized.

No lesions were detected in the spinal cord, medulla, or choroid plexus, while the ganglia show early infiltrative lesions about the blood vessels, and a few instances of necrosis of single nerve cells.

We may consider this series of experiments according as the lesions affect the nervous tissues proper, or as they affect the choroid plexus.

Within the nervous tissues proper, the lesions are, at times, precisely similar to, and indistinguishable from, those produced by intraneural modes of inoculation. However, in certain instances, the lesions present not only resemble those caused by the intraneural modes of inoculation, but differ from them in the extent and degree to which the blood vessels, and those especially in the medulla and pons, are affected. While the degree of perivascular infiltration does not afford a basis of discrimination, a sharp distinction may be drawn between the usual degree of vascular involvement, and the unusual extent in which it occurred in several cases of intravenous injection. What is especially impressive in the latter instances is the diffuse participation of small vessels, down to those of capillary size, in the process and the extension of the infiltrative process from them to the surrounding nervous tissues. Vessels so greatly altered as those under consideration may be considered as contributing to the permeation of the virus from the blood into the tissues. The early lesions of the intervertebral ganglia should be emphasized in this place since they antedate those of the spinal cord and medulla, and extend apparently from vascular lesions.

What appear, however, to be especially important are the changes detected in the choroid plexus, in which infiltrative lesions have hitherto not been observed. That definite lesions of the plexus may occur is clearly indicated by the experiments. As yet no evidence has been obtained of morphological alterations in the ependymal cells, but merely in the blood vessels beneath them. That the secretory functions of the plexus are altered in the direction of greater permeability may be safely assumed, from which it follows that the experiments indicate that when the poliomyelitic infection is induced by the intravenous injection of the virus, there arise, not only the common lesions of poliomyelitis, but also certain additional lesions of the blood vessels and choroid plexus which are of peculiar and distinctive nature.

Since the precise mode of infection in human cases of poliomyelitis may be regarded still as an open question, this criterion of a blood invasion may prove of assistance in the solution of the problem. So far as can be judged from the study of the tissues from several human cases, a corresponding wide-spread vascular involvement to that arising in the experiments would seem not to have occurred.

INTRASPINOUS SERUM PROTECTION.

The data presented confirm and extend the observations already made concerning the passage of the poliomyelitic virus in transit to the central nervous organs from the blood to the cerebrospinal fluid. Since the virus is known to pass successively over several days into the cerebrospinal fluid,[7] in which it seems not to accumulate, but from which it is transferred to the nervous tissues, it was thought that the introduction of a potent immune serum into the meninges at intervals over a number of days would suffice to neutralize the translated virus and thus prevent infection. For this purpose immune serum was available from several monkeys which had recovered from poliomyelitis and had been subsequently reinforced by large subcutaneous injections of the virus.

Earlier experiments had shown that the intraspinous injection of an immune serum is effective under circumstances in which the intravenous injection is not, in delaying or preventing poliomyelitic infection in the monkey. For the next series of experiments it was necessary at the outset to insure that the intravenous injections of the virus would alone induce paralysis, which was accomplished by employing the device of setting up an aseptic meningitis with horse serum, in the animals about to be inoculated.

SERUM AFTER INTRAVENOUS INOCULATION.

Experiment 19.—Macacus rhesus. May 26. Intraspinous injection of 3 c.c. of inactivated 40 per cent. horse serum. May 27. Intravenous injection of 50 c.c. of centrifugalized virus, followed immediately afterwards by an intraspinous injection of 3 c.c. of normal monkey serum. The normal serum was injected intraspinously on May 28, 29, and 30, and, after a two days' interval, on June 2, 3, and 4. June 3. Excitable. June 9. Paralysis of arms and back. June 10. Prostrate; etherized. Typical poliomyelitis.

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[7] Flexner and Amoss, *loc. cit.*

Experiment 20.—Macacus rhesus. May 29. Intraspinous injection of 3 c.c. of inactivated 40 per cent. horse serum. May 30. Intravenous injection of 50 c.c. of centrifugalized virus, followed immediately afterward by an intraspinous injection of 3 c.c. of immune monkey serum. The immune serum injections were repeated May 31, June 1 and 2, and, after a two days' interval, on June 5, 6, and 7. No symptoms developed, and the animal remained normal.

Since these experiments show that the virus may be neutralized by an immune serum in process of passage by way of the cerebrospinal fluid to the nervous tissues, it seemed desirable to ascertain whether a similar neutralization could be affected in a case in which the virus was introduced directly into the meninges by means of lumbar puncture.

SERUM AFTER INTRASPINOUS INOCULATION.

Experiment 21.—Macacus rhesus. May 27. Intraspinous injection of 1 c.c. of emulsion of virus. Two hours later, intraspinous injection of 3 c.c. of normal monkey serum. The injection of normal serum was repeated on May 28, 29, and 30, and, after a two days' interval, on June 2, 3, and 4. June 3. Excitable. June 7. Ataxia; arms and neck weak. June 8. A.M. Arms and back paralyzed; legs weak. P.M. Died. Typical poliomyelitis.

Experiment 22.—Macacus rhesus. May 27. Intraspinous injection of 1 c.c. of emulsion of virus. Two hours later, injected 3 c.c. of immune monkey serum intraspinously. The immune serum injections were repeated on May 28, 29, and 30, and, after a two days' interval, on June 2, 3, and 4. No symptoms developed and the animal remained well.

The preceding experiments show unmistakably that by introducing an immune serum into the subarachnoid spaces, the poliomyelitic virus is capable of being neutralized within the cerebrospinal fluid into which it is directly introduced, or to which it passes in transit from the blood to the nervous tissues. Probably the neutralization in the latter instance is effected at successive stages in process of transfer of the virus to the central nervous organs. Normal serum lacks this power of neutralization.

It may be considered as highly probable that the neutralization is accomplished before any quantity of the virus becomes attached to the nervous tissues themselves. Earlier experiments had shown that when such minute amounts of the virus as one fiftieth to one tenth of a cubic centimeter are inoculated intracerebrally, neutralization is either wholly impossible to accomplish, or is accomplished with very great difficulty even by intraspinous injections of immune serum.[8] Hence the experiments described carry a step further the demonstration that the virus introduced into the blood passes by way of the cerebrospinal fluid to the substance of the nervous tissues in those instances in which paralysis results.

RELATION OF CARMIN TO THE CHOROID PLEXUS AND PERIVASCULAR LYMPHATICS.

That the virus of poliomyelitis is capable, in some instances, of passing from the blood to the cerebrospinal fluid may be considered as demonstrated. This passage takes place probably by way of the choroid plexus and possibly also, to some extent, through the blood vessels in the meninges as well as in the substance of the nervous tissues. Although certain lesions have been detected in the choroid plexus, no morphological alterations have been discovered in the ependymal cells themselves. Hence the question arose whether by the use of pigments the ependymal cells in certain pathological states, including poliomyelitis, might be shown to react in a manner supplying ocular evidences of a disturbance of function.

Carmin is a non-toxic pigment which can be sterilized and suspended in a fine state of subdivision. In this form its introduction into the cerebral ventricles and subarachnoid spaces causes no discomfort in monkeys. Its presence in the meninges and ventricles is followed by an inflammation and rich cellular exudation. The emigrated cells are polynuclear chiefly, but mononuclear cells which take up pigment granules emigrate also. The effects of the carmin injections were studied in normal monkeys, and in monkeys in which an aseptic inflammation had been set up twenty-four hours earlier by means of horse serum, or in which poliomyelitis had been induced by intracerebral inoculation of the virus.

A suspension of the pigment was made in 20 per cent. glycerin, and ammonia was added until solution was complete. This solution was autoclaved and immediately before use was slowly neutralized by the repeated addition of small amounts of sterile 2 N hydrochloric acid, until litmus paper indicated change of reaction. The injections were made under ether anesthesia into the lateral ventricle, the volume of fluid injected being determined by a needle in the lumbar meninges, from which the colored solution was allowed to flow before the injection was stopped.

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[8] Flexner and Lewis, *Jour. Am. Med. Assn.*, 1910, liv, 1780; 1910, lv, 662.

Within twenty-four hours the pigment is distributed over the surfaces of the spinal cord and brain, and within the cerebral ventricles. The base of the brain is deeply and uniformly pigmented. The intervertebral ganglia are either unaffected or mottled with pigment. The nerve roots are visibly pigmented. The choroid plexus appears a vivid red color.

Normal monkeys etherized respectively twenty hours and five days after the injection of the pigment show the inflammatory reaction of the meninges and ventricles mentioned. Interest centers especially in the relation of the pigment to the ependymal cells, choroid plexus, perivascular lymphatics, and intervertebral ganglia. The differences in this respect between the twenty-hour and the five-day specimens are inconspicuous and unimportant.

The pigment appears in two states of division, namely, as excessively minute particles, smaller than many bacteria, and as coarser grains. The latter are contained largely in the mononuclear cells of the inflammatory exudate. The very fine particles have been taken up by the ependymal cells covering the walls of the ventricles and the surfaces of the choroid plexus. Not all, but many of the ependymal cells contain the pigment particles in varying number. The minute and larger grains occur also within cells in the subependymal layer, in close proximity to the ventricles. The latter pigment-containing cells do not seem to have emigrated from the interior of the ventricles, so it is considered probable that the pigment has passed from the ependymal to the subependymal cells. A small quantity of pigment occurs also in the superficial perivascular spaces in the cortex, but not in the spinal cord. The pigment penetrates to the interior of the ganglia with difficulty, along two courses: first the pia capsular investment, second the septa of the nerve roots. About the pigment there is a marked cellular reaction, and pigment-containing leucocytes come to lie against or near nerve cells, but no wide diffusion occurs within the ganglia. The meninges of the brain and spinal cord show a rich cellular exudation containing pigment.

When an aseptic inflammation has been set up previously, and the animal etherized twenty-four hours after the pigment has been injected, the distribution is identical with that described. The single difference noted is a greater amount of pigment within the ependymal and subependymal cells of the ventricles. In the case of an animal in which the pigment was injected during the early paralytic stage of poliomyelitis and which was etherized twenty-four hours later, the cellular accumulations were greater because of the addition of the polynuclear cells to the usual mononuclear infiltration. The choroid plexus of the lateral ventricle showed marked lymphoid, nodular aggregations beneath the ependymal cells, and a rich leucocytic emigration outside. The quantity of pigment taken up by the ependymal and subependymal cells is somewhat greater than that observed in the other instances.

The experiments with the carmin may be interpreted as indicating that the ependymal cells in a living state can be entered by particulate substances. Whether the strictly normal ependymal cells take up and pass on, in the manner described, pigment particles cannot be deduced from the experiments, as the carmin itself causes an inflammatory reaction and consequently may act injuriously upon the cells. Aside from the presence of the pigment, the ependymal cells exhibit no morphological alteration. Apparently a previous inflammation, such as that caused by horse serum and the virus of poliomyelitis, has the effect of rendering the ependymal cells more permeable for the pigment.

PATHOGENESIS OF POLIOMYELITIS.

A consideration of the experiments described in this paper should deal with the question of the pathogenesis of poliomyelitis which they are believed to elucidate.

We are confronted with the problem as to the site of entrance of the virus of poliomyelitis into the human body, as well as the manner in which the specific lesions of the disease are produced. The latter question has already been cleared up in large measure.[9]

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[9] Flexner, *Jour. Am. Med. Assn.*, 1910, lv, 1105. Flexner and Lewis, *Jour. Exper. Med.*, 1910, xii, 227.

It is now sufficiently obvious that the virus possesses affinity for nervous tissues in general, but for no element of these tissues in particular. The constancy with which meninges, blood vessels, interstitial parts, and nerve cells are affected indicates that they all react to the presence of the virus. On the basis of actual observations it cannot be stated that virus is attracted by the nerve cells, either alone or necessarily in advance of the other structures mentioned; while the experiments here recorded show that it is only when the virus is brought to the nervous organs otherwise than by the general blood that the tissues composing them are able readily to remove and attach it to themselves.

This latter fact is a cardinal point, and one from which we may derive valuable information on the pathogenesis and mode of infection of the disease.

It may be regarded as established that all intraneural means of infection are successful, and that the virus travels with more or less ease and certainty along the nerves to the interstices of the central nervous organs, probably utilizing the lymphatic channels of communication. The experiments given in this paper show that the central nervous organs, excepting the intervertebral ganglia, are incapable of removing the virus from the general blood prior to changes induced in the blood vessels and in the choroid plexus. They indicate, also, that in the monkey these preliminary lesions are of a nature that permits of differentiation from the lesions caused by the intraneural modes of infection. The lesions in human cases of poliomyelitis would seem to correspond with those caused by intraneural and not by intravenous inoculation.

In general it should be stated that the intraneural modes of inoculation are effective in proportion to the degree with which they bring the virus into intimate relation with the central nervous tissues. On that account intracerebral inoculation is the most effective, because it not only insures contact between the virus and the mechanically injured tissues, but also because it isolates the virus in the brain tissue, under conditions favorable to multiplication and gradual diffusion into the ventricles and cerebrospinal fluid. Intraspinous injection is somewhat less effective for the reason that a part, and sometimes perhaps all, of the virus may be carried into the general blood before it can reach and become attached to the nervous tissues. Intranasal infection is, in keeping with the general statement made above, more certain in its results than subcutaneous or intrasciatic inoculation, because of the proximity of the short olfactory nerve fiber to the brain tissue. It may fail, because the virus is washed away before it reaches the olfactory fibers and can be carried to the brain. Possibly intraocular inoculation may prove among the most successful, because the virus cannot escape and has only a short distance to travel to the brain; while the vitreous humor may even prove a favorable medium for its multiplication.

In the main, under natural conditions, it is the upper respiratory mucous membrane that would most often become contaminated with the virus, and most readily favor its conveyance to the brain. This series of events is determined by the manner in which the virus is thrown off by the infected body,[10] by the fact of its presence upon the nasal mucosa, even in healthy persons in contact with cases of poliomyelitis,[11] and by the demonstration that it passes, on the whole easily, along the olfactory nerve fibers to the brain, medulla, and spinal cord.[12] Although the virus is conveyed to the nervous organs from without by the lymph, the distribution throughout the nervous system is, in large part at least, effected through the medium of the cerebrospinal fluid. Even when the virus passes from the blood into the nervous organs, it takes the indirect course through the cerebrospinal fluid. This important fact has been established, not only by the finding of the virus by inoculation tests in the cerebrospinal fluid[13] after a blood injection, but also through the prevention of infection by the injection of immune serum into the subarachnoid spaces after lumbar puncture following the intravenous infusion of the virus under conditions insuring infection.

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[10] Flexner, *Jour. Am. Med. Assn.*, 1910, lv, 1105. Flexner and Lewis, *Jour. Exper. Med.*, *loc. cit.*

[11] Flexner, S., Clark, P. F., and Fraser, F. R., *Jour. Am. Med. Assn.*, 1913, lx, 201.

[12] Flexner and Lewis, *Jour. Am. Med. Assn.*, 1910, liv, 1140. Flexner and Clark, *Proc. Soc. Exper. Biol. and Med.*, 1912–13, x, 1. Flexner, *Lancet*, 1912, ii, 1271; *Science*, 1912, xxxvi, 685. Landsteiner and Levaditi, *Ann. de l'Inst. Pasteur*, 1910, xxiv, 833.

[13] Flexner and Amoss. *loc. cit.*

Thus the experimental evidence, which is upheld by observations in human cases of poliomyelitis, supports the view that epidemic poliomyelitis is caused by the entrance into the body of its specific microbic cause or virus, through the upper respiratory mucous membrane to the olfactory lobes of the brain, from which by means of the cerebrospinal fluid it is distributed throughout the substance of the nervous organs; but, since the virus may reach the brain by way of any nervous channel, and even, although with great difficulty, from the blood, it is, of course, possible that in exceptional instances other modes of infection may arise.

SUMMARY.

The virus of poliomyelitis is capable of penetrating the retina without producing apparent injury, to reach the central nervous organs.

The virus injected into the blood is deposited promptly in the spleen and bone marrow, but not in the kidneys, spinal cord, or brain.

Notwithstanding the affinity which the nervous tissues possess for the virus, it is not removed from the blood by the spinal cord and brain until the choroid plexus and blood vessels have suffered injury.

The intervertebral ganglia remove the virus from the blood earlier than do the spinal cord and brain.

An aseptic inflammation produced by an intraspinous injection of horse serum facilitates and insures the passage of the virus to the central nervous organs, and the production of paralysis. The unaided virus, even when present in large amounts, passes inconstantly from the blood to the substance of the spinal cord and brain.

When the virus within the blood fails to gain access to the central nervous organs, and to set up paralysis, it is destroyed by the body, in course of which destruction it undergoes, as a result of the action of the spleen and, perhaps, other organs, diminution of virulence.

The histological lesions that follow the intravenous injections of the virus in some but not in all cases differ from those which result from intraneural modes of infection.

In escaping from the blood into the spinal cord and brain, the virus causes a lymphatic invasion of the choroid plexus and widespread perivascular infiltration, and from the latter cellular invasions enter the nervous tissues. A similar lymphoid infiltration of the choroid plexus may arise also from an intracerebral injection of the virus.

The histological lesions present in the central nervous organs in human cases of poliomyelitis correspond to those that arise from the intraneural method of infection in the monkey.

The virus in transit from the blood through the cerebrospinal fluid to the substance of the spinal cord and brain is capable of being neutralized by intraspinous injection of immune serum, whereby the production of paralysis is averted.

Carmin in a sterile and finely divided state introduced into the meninges and ventricles sets up an aseptic inflammation, but is quickly taken up by cells, including ependymal cells. When an aseptic inflammation has been previously established by means of horse serum, or when the nervous tissues are already injured by the poliomyelitic virus, the pigment appears to enter the ependymal cells more freely.

The experiments described support the view that infection in epidemic poliomyelitis in man is local and neural, and by way of the lymphatics, and not general and by way of the blood. Hence they uphold the belief that the *infection atrium* is the upper respiratory mucous membrane.

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EXPERIMENTS ON THE RÔLE OF LYMPHOID TISSUE IN THE RESISTANCE TO EXPERIMENTAL TUBERCULOSIS IN MICE.*

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In the reaction to tuberculous infection the part played by the endothelioid cell or endothelial leucocyte (Mallory) has received much attention in recent years. This is probably due to the early appearance of these cells in the process of formation of the tubercle and because of their well known phagocytic activities.[1] The other conspicuous element in tubercle formation, the lymphoid cell, probably because of its later appearance and its lack of phagocytic power, has been almost entirely neglected. The small round cell infiltration about the tubercle has indeed been regarded by most observers as of very secondary importance and is often referred to as resulting probably from tissue destruction.

In spite of the lack of interest among pathologists concerning the rôle of the lymphocyte in the tuberculous process, attention has been called by a number of clinicians to the prognostic importance of the lymphocytes in the circulating blood.[2] These observers have noted that in rapidly fatal miliary tuberculosis the lymphocytes fall, often to below 10 per cent. of the circulating white cells, whereas in patients with early, healed, or healing tuberculous lesions these cells are increased, sometimes forming more than 50 per cent. of the total leucocytes.[3] Moreover, it is well known that in acute rapidly fatal

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* Received for publication, August 18, 1914.

[1] For the literature see Goldmann, E. E., Neue Untersuchungen über die äussere und innere Sekretion des gesunden und kranken Organismus im Lichte der "vitalen Färbung," Tübingen, 1912, 52; and Evans, H. M., Bowman, F. B., and Winternitz, M. C., *Jour. Exper. Med.*, 1914, xix, 283.

[2] For the literature, see Brecke, A., in Brauer, L., Schröder, G., and Blumenfeld, F., Handbuch für Tuberkulose, Leipzig, 1914, i, 581.

[3] Wack, P., *Deutsch. Arch. f. klin. Med.*, 1914, cxv, 596.

miliary tuberculosis relatively few lymphocytes occur in the individual tubercles, while in the subacute form where a higher resistance of the individual may be assumed, the tubercles contain large numbers of lymphoid cells. These facts have a close analogy in the reaction of the polynuclear leucocytes in certain infections against which they are presumed to form the chief resisting factor.

LYMPHOCYTES AS FACTORS OF RESISTANCE.

Until recently, the round cell infiltration occurring about slowly growing or healing cancer, about cancer grafts in immunized animals, about failing tissue grafts in unsuitable or resistant animals, has been assigned, as in tuberculosis, a secondary rôle. The lymphoid elements here, too, are supposed to be present as a result of tissue disintegration. However, it has been shown that in the case of tissue grafts, the lymphocytes in all probability are the chief agents in causing the destruction of the introduced tissue. The chick embryo normally has no resistance against the growth of implanted tissues from a foreign species, and likewise shows a total absence of the round cell infiltration about the graft.[4] When, however, the chick embryo is provided with a graft of adult chicken lymphoid tissue, it becomes as resistant as the adult to the growth of implanted tissue from a foreign species, and, like the adult, shows an intense infiltration of small round cells about the foreign graft.[5] Furthermore, if the lymphoid system of an adult animal is depleted by means of X-ray, the animal loses its power of resistance to heterologous tissue and an implanted tissue from a foreign species will then grow readily. It is significant that in such animals there is a total absence of the round cell infiltration about the edges of the graft always present in resistant untreated animals.[6]

In the light of these results it seemed probable that the lymphoid cell might play a more important rôle in the resistance to certain infections than had previously been supposed. The fact of its presence in the reaction to tuberculous infections, its variation in the blood with the condition of the tuberculous individuals, and the recent experiments of Lewis and Margot[7] suggested the advisability of investigating the part played by the lymphocyte in the resistance to tuberculosis. Lewis and Margot made the observation that rats and mice experimentally infected with tuberculosis developed large spleens. Splenectomized animals, however, lived longer after inoculation than normal animals. This anomalous result seemed difficult to explain. It is interesting to note in this connection that the inoculations were never made in less than two weeks after the splenectomy and were usually done in the third week. At this period the lymph glands and the lymphoid tissue of the body have shown considerable hypertrophy and many animals show a great increase over normal in the circulating lymphocytes.[8]

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[4] Murphy, Jas. B., *Jour. Exper. Med.*, 1913, xvii, 482.

[5] Murphy, Jas. B., *idem,* 1914, xix, 513.

[6] Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1914, lxii, 1459.

THE EFFECT OF X-RAY ON LYMPHOID TISSUE.

Heineke has shown that X-ray has an almost specific and immediate destructive action on the lymphoid system, and in small doses seems to have little, if any, effect on other cells of the body.[9] These results have been adequately confirmed by other observers. We have found that by carefully regulated doses of X-ray, repeated at intervals, a gradual atrophy of the lymphoid tissue may be accomplished without any appreciable effect on the other tissues, or on the general health of the animal.

TUBERCULOUS INFECTIONS IN X-RAYED MICE.

This specific effect of X-ray on the lymphoid tissue offers an excellent experimental method for testing the value of the lymphocyte in various conditions. Animals whose lymphoid tissue has been destroyed should be highly susceptible to those infections against which the lymphocytes play a part in the defense, while their resistance to those infections met by the polymorphonuclear leucocytes should be unaffected. With this idea in view the following experiments were planned.

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[7] Lewis, P. A., and Margot, A. G., *Jour. Exper. Med.*, 1914, xix, 187.

[8] Dr. Linda B. Lange has made a series of differential counts on mice before and at intervals after splenectomy. These will probably be reported later.

[9] Heineke, H., *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1905, xiv, 21.

Experiment I.—Fifty mice of about the same age and size were selected. These were divided into lots of ten each and subjected to the following treatment.

A. Ten mice were splenectomized and then given a daily 5-minute exposure to X-ray for 2 weeks. Average dosage less than ¼ unit (Holzknecht scale), average penetration No. 6, milliamperes 3-4. Spark gap 1 to 1½ inches.

B. Ten normal mice were given daily exposures to X-ray for 2 weeks in the same dosage as group A.

C. Ten mice were splenectomized on the date of inoculation 4 to 9 hours before this procedure.

D. Ten mice were splenectomized 8 to 10 days before the date of inoculation.

E. Ten normal animals served as controls.

All the animals were in good condition at the time of inoculation.

They were divided into small groups in order to prevent the spread of epidemics if any should develop, and were then inoculated with an emulsion of a 6 weeks' glycerin veal bouillon culture of bovine tubercle bacilli, each animal receiving a dose of 1 mg. of dry tubercle bacilli in 0.8 c.c. normal salt solution.[10] As control to the X-ray effect numerous mice have been given the same or larger doses and have shown no bad effects while under observation for several weeks afterward.

TABLE I.

Animals.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidney.
Group A	7.3 dys.	100		100	40	80	100
Group B	7.0 dys.	100	100	100	60	80	100
Group C	9.1 dys.	100		100	50	80	100
Group D	19.7 dys.	100		100	90	100	100
Group E	18.5 dys.	100	100	100	80	70	100

Group A = 10 splenectomized mice given 14 daily exposures to X-ray. Group B = 10 normal mice given 14 daily exposures to X-ray. Group C = 10 mice splenectomized a few hours before inoculation. Group D = 10 mice splenectomized 8 to 10 days before inoculation. Group E = 10 normal mice as controls.

Table I shows the average number of days the various groups of animals lived and the percentage of animals showing tubercle bacilli in the blood, peritoneal exudate, and the various organs. The two X-ray groups, A and B, lived on an average about seven days after the inoculation, and group C, splenectomized a short time before inoculation, averaged only about nine days. Group D, the animals splenectomized about ten days previous to the inoculation, averaged 19.7 days which is a little more than a day longer than the normal group E, which survived 18.5 days as an average. The widespread distribution and great number of organisms leave little doubt that the tubercle bacilli were the cause of death. To rule out epidemics of mouse typhoid, cultures were always taken from the heart's blood and the character of any organisms obtained was studied.

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[10] This culture was kindly provided by Dr. Paul A. Lewis of the Henry Phipps Institute. It is termed by him Bovine C. The organisms were dried by pressing between filter papers, weighed, and then made into an emulsion by long grinding in normal salt solution.

Experiment 2.—This experiment confirms and adds a further control to experiment 1. The groups of mice used were as follows:

A. Eight mice were splenectomized and given 12 daily exposures to X-ray in the same dosage as in experiment 1.

B. Nine normal mice were given the same X-ray exposures as those in group A.

C. Sixteen small normal mice were given 17 daily exposures of X-ray of the same intensity as those in groups A and B. The exposures were discontinued 4 weeks before the inoculations were made.

D. Ten mice were splenectomized 3½ weeks before inoculation.

E. Ten normal mice.

All these animals except 8 of group C were given 2 mg. of a 9 weeks' old culture of bovine tubercle bacilli of the same strain as that used in the first experiment. These animals were isolated in individual glass jars so as to prevent the spread of epidemics, should any develop.

TABLE II.

Animals.	Average time of survival.	Percentage of animals with tubercle bacilli in various organs.					
		Exudate.	Spleen.	Liver.	Heart's blood.	Lungs.	Kidney.
Group A.........	8.4 dys.	100		80	20	40	40
Group B.........	9.7 dys.	100	100	66	11	44	55
Group C.........	7.1 dys.	100	88	71	42	57	100
Group D.........	26.0 dys.	75		87	25	37	62
Group E.........	23.3 dys.	100	100	80	10	90	70

Group A = 8 mice splenectomized and given 12 daily exposures to X-ray. Group B = 9 normal mice given 12 daily exposures to X-ray. Group C = 16 mice given 17 daily exposures to X-ray; 8 of them were inoculated 4 weeks after X-ray was discontinued. Group D = 10 mice splenectomized 3½ weeks before inoculation. Group E = 10 normal mice.

Table II shows the death rate and distribution of the organisms. The age of the culture explains the longer survival of the animals even with twice the dose used in the first experiment. Groups A and

B, the splenectomized X-rayed and the normal X-rayed animals, lived an average of 8.4 and 9.7 days, respectively, after inoculation. Of the sixteen mice in Group C, the life of the eight which were inoculated averaged only 7.1 days after inoculation. Although these animals had had a month to regenerate their lymphoid tissue, they still showed a completely depressed resistance. As a matter of fact, the autopsies showed the spleens and lymph glands to be atrophic, with little sign of regeneration. The early death in these animals compared with the other X-rayed animals may perhaps be due to the fact that they were much smaller mice and had been given more frequent exposures to X-ray. Eight mice of this lot which were not inoculated but kept as a control to X-ray effect are still living and in perfect condition almost three months after X-ray treatment. The mice splenectomized three and a half weeks before inoculation, in agreement with the results of Lewis and Margot, lived longer than the normal animals.

The experiments were planned with the idea of testing the resistance of the animals to tuberculosis when the amount of the lymphoid tissue was varied. The X-rayed animals are at the bottom of the scale, having a system greatly depleted, and they were the first to die of the disease. Next come those splenectomized shortly before the inoculation; these may be considered as having a reduced amount of lymphoid tissue. The animals inoculated eight to ten days after splenectomy, having an active proliferation of the lymphoid cells in the glands and elsewhere, at this stage apparently have about the same resistance as the normal animals. The animals, however, splenectomized three or more weeks before inoculation outlive the normal animals by a number of days and probably represent an increased activity of the defensive agents.

DISCUSSION.

The question naturally arises: Do these treatments cause variations in factors other than the lymphocyte, which might play a part in the resistance to tuberculosis? X-ray in the amount used in these experiments does not affect the general health of the animal. The polymorphonuclear leucocytes are not decreased in number and may be increased. The X-rayed animals have a normal resistance to certain infecting agents against which these cells form the defense and in some cases they may even have an increased resistance. The circulating large mononuclear cells are not appreciably affected. As evidence that the endothelioid cells are not destroyed, great numbers of these are found in the spleen and lymph glands after X-ray treatment, actively phagocyting the remains of the lymphocytes.[11]

The well known association of the lymphocytes with tuberculous lesions tends to support the conclusions indicated by these experiments. In the acute miliary type of the disease where it may be supposed that little resistance is being offered, the lymphocytes are relatively few in the tubercle, while in the subacute miliary tuberculosis these cells occur in masses about the lesion. As has already been mentioned, the lymphocytes in the blood fall in the rapidly advancing cases, while individuals with localized and well controlled lesions will show a marked increase in the circulating lymphocytes. It would seem, therefore, that these facts taken in conjunction with our experiments strongly suggest that the lymphocyte plays an important rôle in the animal's resistance to tuberculosis.

SUMMARY:

Mice either normal or splenectomized after exposure to X-ray are markedly more susceptible to bovine tuberculosis than are normal animals. Animals splenectomized a short time prior to inoculation are also more susceptible than normal, while those splenectomized eight to ten days before inoculation have about the same resistance as normal. The mice splenectomized three to four weeks before inoculation have a resistance increased over the normal, as has already been shown by Lewis and Margot. As X-ray in the doses used apparently affects only the lymphoid tissue and as the hypertrophy of the remaining lymphoid tissue after splenectomy is so rapid that the circulating lymphocytes may be much above the normal by the third week, it is concluded that this evidence, taken with the well known association of the lymphocytes with tuberculous lesions, points strongly to the lymphocyte as an important agent in the defensive mechanism against tuberculosis.

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[11] Heineke, H., *loc. cit.* See illustrations.

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ON THE CAUSE OF THE LOCALIZATION OF SECONDARY TUMORS AT POINTS OF INJURY.*

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PLATES 18 TO 20.

The localization of secondary tumors at points of injury has been so often remarked upon that it is unnecessary to cite specific instances. The cause for the phenomenon is unknown. Lubarsch[1] has shown that mouse tumors may be made to localize secondarily in the liver, about splinters implanted in this organ, but he did not attempt to explain the finding.

There are a number of observations which make it evident that the development of tumor metastases from tumor emboli is conditional upon a special set of circumstances. Schmidt[2] has shown that a large proportion of tumor cells cast off into the blood stream may die without giving rise to metastases. He found that the pulmonary arterioles of patients with visceral cancer often contain many tumor emboli that are dead or dying. The fact has been repeatedly noted that rats and mice inoculated intravenously with tumor fragments seldom develop growths in the lungs despite the fact that the same material causes tumors when implanted subcutaneously. So too in these animals the intraperitoneal inoculation of active tumor material yields comparatively few takes.

It has seemed to us possible to study some of the factors concerned in the secondary localization of tumors by means of experiments involving tumor localization on the lining of the peritoneal cavity. According to Schmidt, the intima of the blood vessels constitutes the essential barrier to invasion of the tissue by the cells of tumor emboli. The peritoneal lining presents much the same structural

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* Received for publication, August 1, 1914.

[1] Lubarsch, O., *Med. Klinik*, 1912, viii, 1651.

[2] Schmidt, M. B., Die Verbreitungswege der Karzinome und die Beziehung generalisierter Sarkome zu den leukämischen Neubildungen, Jena, 1903.

features as the intima of the blood vessels, namely, a single layer of flattened cells covering a connective tissue. When tumor fragments are injected into the blood stream they are often surrounded by a thrombus and furthermore are so widely scattered that their fate is difficult to follow. Both these difficulties are avoided by the use of the peritoneal cavity.

The first question to present itself is that of the nature of the forces which prevent, or at least hinder, the take of tumor fragments inoculated intraperitoneally. Is there an immunity reaction in which the fluids of the cavity are concerned, or merely a resistance offered by an intact serosa? To test the point we have injured the peritoneum of mice by mechanical means, afterwards inoculating a tumor. For the purpose finely ground diatomaceous earth (*Kieselguhr*) has been used and an adenocarcinoma known in our laboratory as Mouse Tumor 33. Mouse Tumor 33 grows in discrete masses, seldom infiltrating or becoming diffuse (figure 1). Because of this it was especially suitable for the work.

Experiment 1.—Fifteen mice were injected intraperitoneally with 0.25 c.c. of finely ground, sterile *Kieselguhr* suspended in Ringer's solution, and three days later with 0.1 c.c. of a suspension of very small fragments of mouse carcinoma in Ringer's solution. Control animals were injected with the tumor suspension only.

At the end of two weeks nine of the mice receiving both *Kieselguhr* and carcinoma remained. They were killed and carefully autopsied. Seven had tumors in the subcutaneous tissue and on the peritoneum where the injecting needle had been thrust through. In all of these, nodules were found scattered throughout the peritoneal cavity, and in several the liver, spleen, and kidneys were involved. The remaining two animals were negative.

Eight controls survived the two weeks. Six had tumors along the track of the inoculating needle similar to those in the experimental animals, but in only one had dissemination taken place in the peritoneal cavity.

This experiment and others similar show that an acute injury to the peritoneum, mechanically caused, renders it more suitable for the lodgment and growth of mouse tumor. Microscopic examination of the nodules on the parietal peritoneum, in the mesentery, and on the surface of the liver and spleen of the experimental animals has revealed an interesting condition. The particles of *Kieselguhr* are not distributed evenly but lie in aggregates here and there in the midst of a layer of newly formed and very cellular connective tissue covered with endothelium. The tumors are in general definitely localized to these areas (figure 2). Many small discrete clumps of neoplastic cells are to be noted lying embedded in the reactive tissue and covered with endothelium. In the case of the larger, more diffuse tumors the association with the *Kieselguhr* is also evident (figure 3).

Although the results are clear cut they do not enable one to conclude whether it is damage to the connective tissue or to the endothelium that renders the peritoneal surface susceptible. For the inoculations were made at a time when the *Kieselguhr* had but just cut its way through the endothelium. In a later series of experiments two weeks were allowed to elapse between the *Kieselguhr* inoculation and the injection of the tumor, in order that the endothelium might have opportunity for complete repair. Sections show that after this time the *Kieselguhr* is enclosed in small discrete accumulations of quiescent connective tissue, completely covered by endothelium.

Experiment 2.—Ten mice were injected intraperitoneally with 0.25 c.c. of finely ground *Kieselguhr* suspended in Ringer's fluid, followed two weeks later by 0.1 c.c. of a suspension of mouse carcinoma. Ten control animals received the tumor suspension only.

Five of the mice that received the *Kieselguhr* and carcinoma were alive two weeks after the injection of the latter. They were killed and examined at this time. In four, tumors had developed along the track of the injecting needle while in the remaining one the tumor had failed to take. In all four susceptible animals tumor nodules were found throughout the peritoneal cavity.

Autopsy of the seven surviving controls revealed the following: Four had growths along the track of the needle and in three the tumor had failed to take. In one of the susceptible animals a nodule was present in the mesentery directly opposite the point of injection. In the others the peritoneal lining was normal.

This experiment and others of the same sort show that an injured peritoneal lining remains favorable to tumor implantations after the endothelium has repaired itself completely. Experiments in which lycopodium spores were used as the foreign body have given identical results. Unlike the *Kieselguhr* the spores do not penetrate but are rapidly surrounded by endothelial cells and later encapsulated by connective tissue (Marchand). As a rule quite a number of them are found lying together in a web of newly formed connective tissue. The little nodules so composed are covered with endothelium (figure 4). They offer a most favorable locus for tumor implantation (figure 5). We feel justified in concluding that it is the derangement of the connective tissue, rather than of the endothelium which renders an injured peritoneal lining favorable to the lodgment and growth of tumor fragments.

The damage caused by *Kieselguhr* or by lycopodium is punctate in character, but it is wide-spread and might conceivably alter the ability of the peritoneum to elaborate immune substances, or to form the medium of their passage. It has seemed necessary, therefore, to perform experiments involving a relatively insignificant and sharply localized damage to the peritoneum. One or several small, sterile glass rods, rounded at the ends, were introduced into the peritoneal cavity of mice through a trocar and followed later by a tumor suspension.

Experiment 3.—Two or three glass rods about 1 mm. in diameter and 8 mm. long were introduced into the peritoneal cavity of each of ten mice. Two weeks later the animals were inoculated intraperitoneally with 0.1 c.c. of a fine suspension of mouse carcinoma. Ten control animals also received the tumor material at this time.

Six of the experimental animals were alive after two weeks. Four had a tumor in the track of the inoculating needle. In all four, intraperitoneal tumors were found situated next to the glass rods and there only (figure 6). The remaining two mice were negative.

Three of the controls survived two weeks. Two had tumors in the injection track and the other was negative. In one of the susceptible animals a few tiny, discrete tumor nodules were found on the mesentery.

The injury caused by a smooth, glass rod where it lies in contact with the peritoneum renders this latter favorable to tumor implantation. An alternative explanation, that the localization of the neoplasm was due to an accumulation of tumor fragments in a dead space about the rods, fails, because there was no dead space, the rods being closely enveloped in mesentery.

Evidently the resistance manifested by a healthy peritoneum to the lodgment and growth of tumor fragments is not due to a general immunity reaction, but is referable to the physical characters of the lining membrane.

In the light of our results, one may ask whether the factor of injury may not play a part, heretofore unrecognized, in the peritoneal dissemination of certain visceral tumors of human beings. It is true that some growths are so malignant that fragments sown on an intact serosa can successfully lodge and grow. This has been noted of certain rat and mouse tumors as well. But at the other end of the scale there are visceral growths which fail to localize on the peritoneal lining, although fragments of them must be distributed to it. With tumors of intermediate malignancy may it not be that the first fragments that are cast off die, and, causing inflammation, render the peritoneal lining more susceptible for future implants? To test the point mice were inoculated intraperitoneally with bits of killed tumor and later with particles of the living growth.

Experiment 4.—Fifteen mice were injected intraperitoneally with 1.0 c.c. of coarse particles of killed mouse carcinoma suspended in Ringer's fluid. The suspension had been heated in the water bath at 55° C. for 15 minutes, a temperature sufficient to kill the cells of the tumor. Three days later each animal received a second inoculation of 0.06 c.c. of a suspension of living tumor fragments. Fifteen control mice were also inoculated at this time.

Seven of the experimental animals were alive two weeks later.' In all, tumors had developed in the inoculation track. In six, there were nodules throughout the abdominal cavity. In the remaining animal two discrete nodules were found in the mesentery.

Ten controls survived. Four were completely negative as regards tumor. Six showed tumors in the injection track. Three of these had one or two tiny, sharply circumscribed growths in the mesentery, and a fourth showed many disseminated growths. In the other two the peritoneal lining was healthy looking.

Jobling[3] has shown that the intraperitoneal injection of a suspension of rat carcinoma killed by heat may increase the susceptibility of animals for later subcutaneous implantations of the same tumor. The results of the present experiment might be referred to a similar hypersusceptibility, were it not that in other of our experiments with identical intraperitoneal findings there is no evidence for this, tumors developing along the track of the injecting needle in about the same proportion of control animals and those injected with the killed suspension. Furthermore, special tests have shown that killed suspensions of Mouse Tumor 33 do not induce hypersusceptibility.

Since dead tumor fragments in contact with the peritoneal lining render this latter more suitable for the lodgment and growth of tumor cells, it seems probable that the peritoneal dissemination of some human tumors may indeed come about through the death of the first tumor fragments cast off, and the reaction thus caused.

[3] Jobling, J. W., *Monographs of The Rockefeller Institute for Medical Research*, 1910, No. 1, 52.

In what way does an injury to the peritoneal lining, or, more precisely, to the subendothelial connective tissue favor tumor localization and growth? The observations of Schmidt,[4] already mentioned, offer a suggestion. Schmidt found that tumor cells which had lodged in the pulmonary arterioles were unable to penetrate the vascular endothelium directly, although they might proliferate and ramify within the lumen of the vessel. Whether they ultimately invaded the surrounding tissue depended upon whether they were supplied with a supporting stroma by the subendothelial connective tissue. Now, in the case of the peritoneal lining we have found that the reactive changes caused by an injury to the subendothelial connective tissue greatly favor the lodgment and growth of bits of tumor. It seems possible that the stroma for a tumor fragment might be elaborated with especial ease by a connective tissue in course of proliferation as the result of an injury. As bearing on the point, we have compared the growth *in vitro* of connective tissue reacting to the presence of a foreign body with the growth of normal connective tissue from the same region. Implantations were made into chicken plasma of bits of tissue from about glass rods embedded for various periods in the breast muscle of fowls. Fowls were chosen because they are extremely resistant to local infection and because their plasma can be readily handled.

Experiment 5.—Eight sterile, smooth glass rods about 1.5 by 8 mm. were inserted on successive days by means of a trocar into the pectoral muscles of each of three fowls. The rods were marked for identification. When they had been in place for 1, 2, 3, 4, 6, 8, 10, and 12 days, respectively, the fowls were killed and many small pieces of tissue from about the rods were implanted in chicken plasma and incubated at 41° C. No infection had occurred. Pieces of normal connective tissue, of fascia, and of muscle from near by were also implanted and incubated. The results were striking. No growth occurred from the control fragments or from those about rods that had been in place only one day. There was marked and very prompt emigration of large, rounded ameboid cells from the fragments that had been next to the rods for two or three days. In a few instances there was a definite growth of fibroblasts as well. The pieces removed from about rods that had been in place 4, 5, 6, and 8 days showed a profuse connective tissue growth which began after only a few hours of incubation. In fact, the rapidity and amount of this growth compared very favorably with that of some sarcomata and of embryonic tissue. By the twelfth day the tissue encapsulating the rods had become quiescent and little growth was obtained from it.

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[4] Schmidt, M. B., *loc. cit.*

The experiment shows conclusively that connective tissue reacting to the injury caused by the presence of a foreign body has a proliferative energy greater than the normal. Furthermore, its growth in plasma takes place without that latent period which Carrel has described for normal adult connective tissue.[5] The rounded cells that emigrated from the tissue which had been two and three days in contact with the glass rods were doubtless wandering cells attracted by the foreign body; and the true growth that took place obviously came about by the proliferation of the many fibroblasts present in the reactive tissue. From such results it seems highly probable that connective tissue, reacting to an injury, is in a condition to elaborate the stroma for a tumor more rapidly and abundantly than normal tissue.

Altogether, the findings seem to us to indicate that the secondary localization of tumors at points of injury is referable to the presence at such points of a very cellular connective tissue which may come more readily than the normal to the support and nourishment of the tumor cells. A number of facts in the literature may be taken to support this view. To mention only two of them, Loeb and Sweek[6] have described epitheliomata of which the sluggish course was apparently referable to the resistance offered by an inert connective tissue; and Levin[7] has shown that the Flexner-Jobling rat tumor, inoculated into the normal testicle of rats and into testicles previously injected with *Scharlach R* and ether water, will grow only in the latter. Levin ascribes this finding to some chemical influence inducing a "precancerous state" in the testicle. It would seem more likely that it is referable to the presence of a highly labile connective tissue capable of immediate and active proliferation in support of the tumor. The rapid spread of tumor tissue in a wound is explicable on the same basis.

SUMMARY.

The cause of the frequent localization of secondary tumors at points of injury is not known. Our work deals with this problem.

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[5] Loeb has found that regenerating kidney grows better than the healthy organ *in vitro* (Loeb, L., *Anat. Rec.*, 1912, vi, 109).

[6] Loeb, L., and Sweek, W. O., *Jour. Med. Research*, 1913, xxviii, 235.

[7] Levin, I., *Jour. Exper. Med.*, 1912, xv, 163.

For the experiments the peritoneal cavity has been employed as offering relatively uncomplicated conditions, and the fate of mouse tumor brought into contact with a peritoneal lining injured in various ways has been studied.

The injection of a suspension of mouse tumor into a healthy peritoneal cavity has little success as a rule compared with a similar injection into the subcutaneous tissue. We have found that the resistance of the peritoneal lining thus indicated can be largely if not completely abolished by the preliminary injection of a mechanical irritant (*Kieselguhr,* lycopodium). That the change thus brought about is independent of general immunity phenomena is shown by the fact that a local injury renders susceptible the part of the peritoneum immediately affected and that part only. Special tests show that the factor important in rendering the peritoneum more susceptible is the injury to the subendothelial connective tissue. Susceptibility persists after the endothelium has regenerated over the reacting connective tissue.

Schmidt has found that the cells of tumor emboli in the pulmonary arterioles are able to penetrate the endothelium of the vessel only after they have been provided with a stroma from the subendothelial connective tissue. Our findings are easily explained on the basis thus suggested. A connective tissue highly cellular and perhaps still proliferating as the result of injury may well elaborate the stroma for a tumor more rapidly than normal connective tissue. Tests of growth *in vitro* support this idea. Connective tissue reacting to an injury grows profusely and almost immediately when incubated in plasma, whereas normal tissue from the same region shows usually no growth whatever.

Dead tumor fragments in contact with the peritoneum cause a change favorable to the lodgment and growth of later tumor fragments. It seems not improbable that the peritoneal dissemination of certain human neoplasms may be accomplished indirectly through the death of the first tumor fragments cast off.

Our observations have been purposely confined to the effects of injury on the peritoneal lining ; but they seem to afford the basis for a generalization. The secondary localization of tumors at points of injury may be attributed with good reason to the presence at such points of an active connective tissue capable of elaborating a stroma rapidly and abundantly. For it is the proliferation of the subendothelial connective tissue to form a supporting stroma that determines the fate of free tumor cells, whether these lie on the peritoneum or within a vessel.

EXPLANATION OF PLATES.

PLATE 18.

FIG. 1. A nodule of Mouse Tumor 33 on the serous coat of the intestine, showing the discrete, uninvasive character of the growth. The nodule is largely necrotic.

FIG. 2. A portion of the parietal peritoneum and abdominal muscle of a mouse receiving an injection of *Kieselguhr* and three days later one of mouse tumor. The animal was killed two weeks after the second injection. On the peritoneal surface are two nodules of reactive tissue containing *Kieselguhr* and a third such nodule in which the tumor has localized.

PLATE 19.

FIG. 3. A higher magnification of a portion of the tumor nodule shown in the preceding figure. The *Kieselguhr* is indicated by the arrows.

FIG. 4. Lycopodium spores on the surface of the spleen. They lie grouped together, are embedded in connective tissue, and covered by endothelium.

PLATE 20.

FIG. 5. Portion of a tumor associated with the reactive tissue about lycopodium spores. To be compared with figure 4. Part of the abdominal muscle is shown.

FIG. 6. Viscera of a mouse receiving an intraperitoneal injection of tumor fragments two weeks after the introduction into the peritoneal cavity of three small glass rods. There are discrete tumors (*a* and *b*) in the vicinity of the rods but none elsewhere.

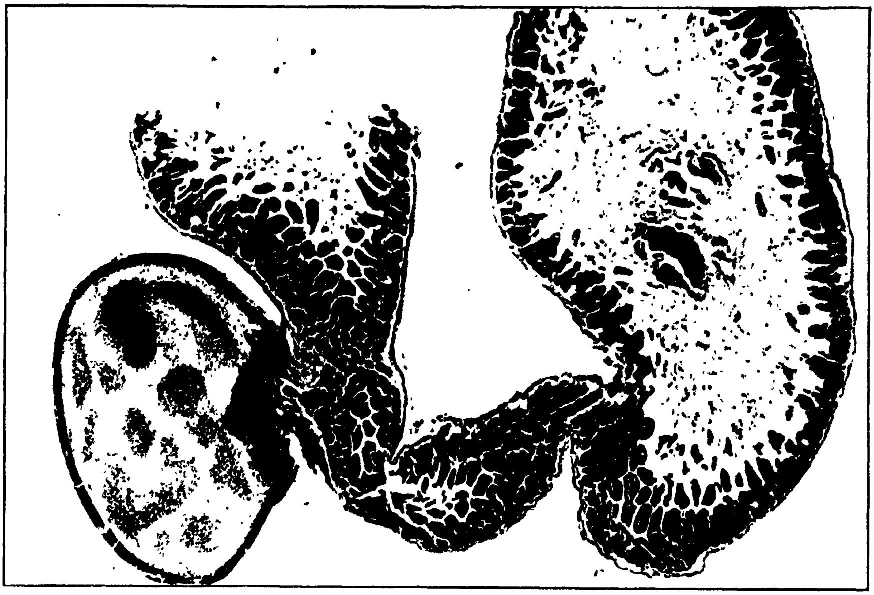


FIG. 1.

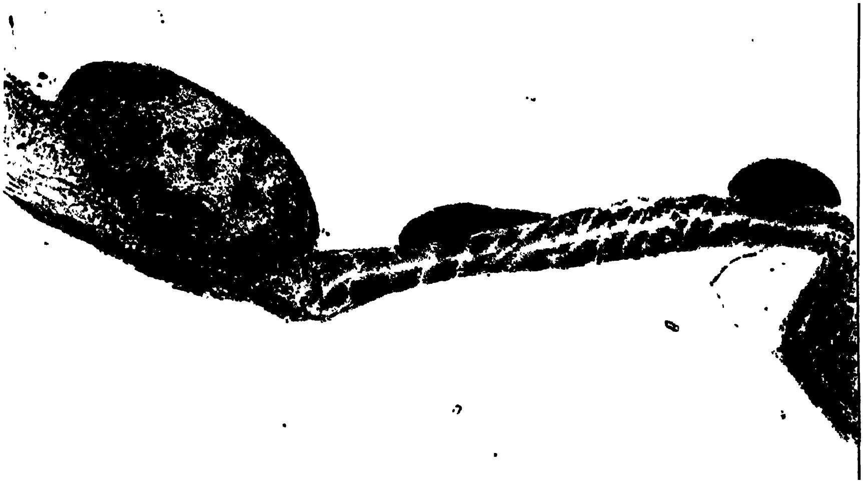


FIG. 2.

(Jones and Rous: Localization of Secondary Tumors.)

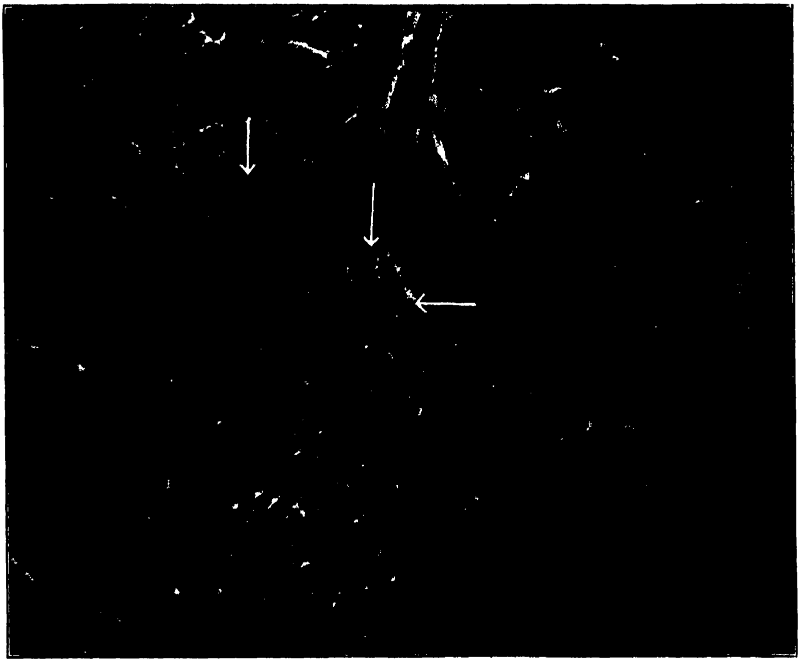


FIG. 3.

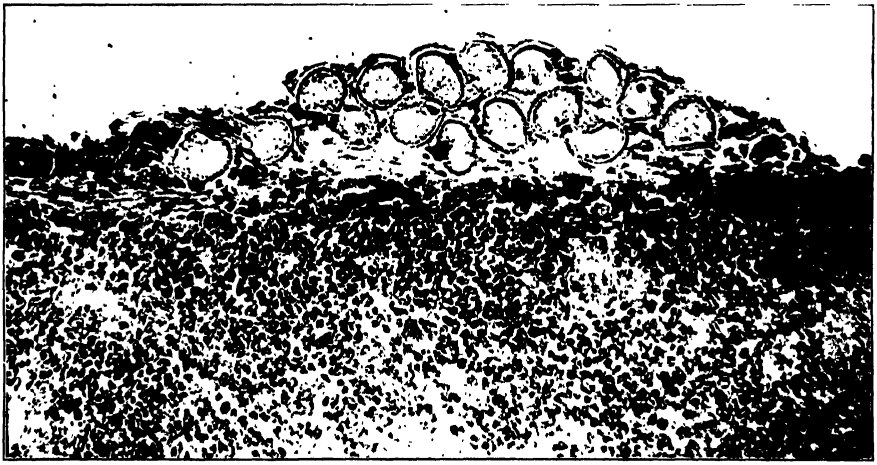


FIG. 4.

(Jones and Rous: Localization of Secondary Tumors.)

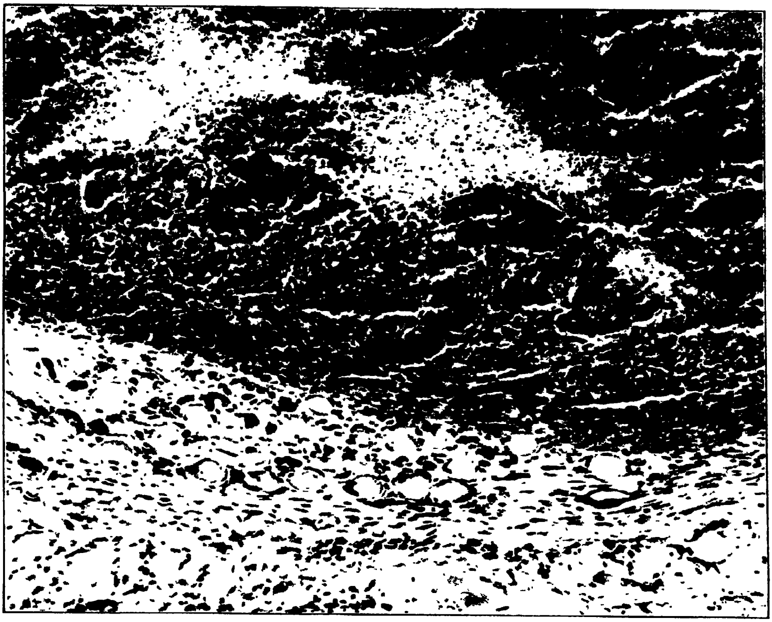


FIG. 5.

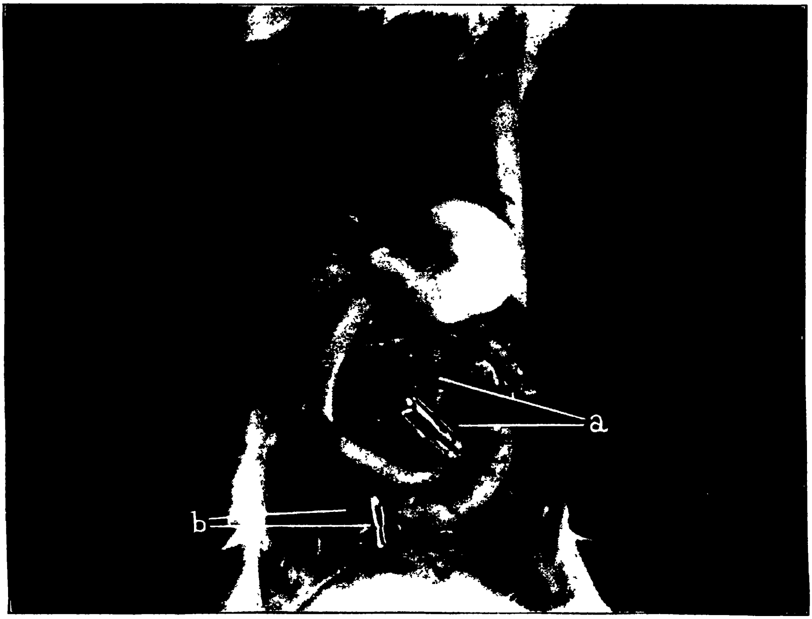


FIG. 6.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, October 1, 1914, Vol. xx, No. 4, pp. 413–418.]

ON THE GREATER SUSCEPTIBILITY OF AN ALIEN VARIETY OF HOST TO AN AVIAN TUMOR.*

BY PEYTON ROUS, M.D., AND LINDA B. LANGE, M.D.

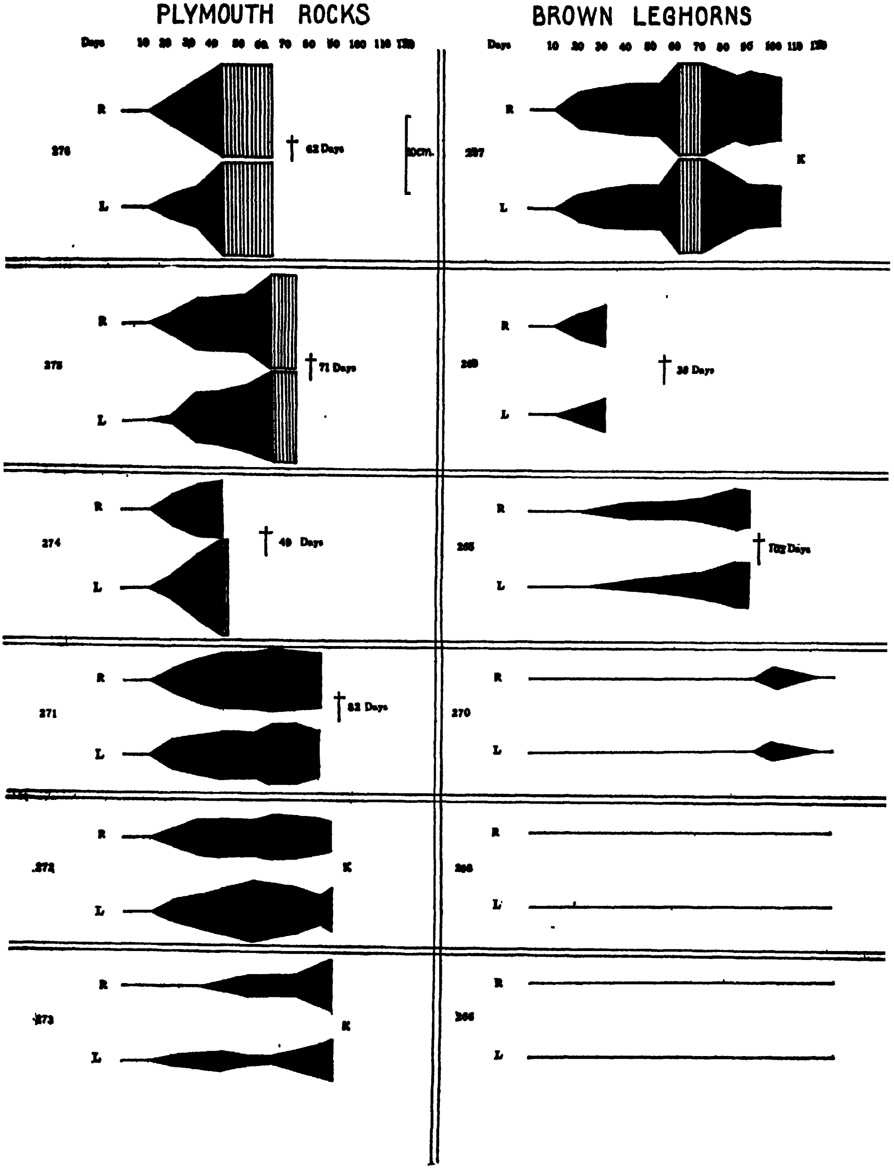
(From the Laboratories of The Rockefeller Institute for Medical Research.)

It has been the general experience of workers with transplantable tumors that they are most readily propagated in animals of the variety in which they appear as spontaneous growths (Jensen, Loeb). Some tumors are so specific in their demands as to grow only in individuals from certain sources and not in others of the same race. A spindle-celled sarcoma of the fowl studied in our laboratory was successfully transferred at first only to blood relations of the original host. In fowls of the same variety, but unrelated, it did not grow. The purpose of the present paper is to record an instance in which the behavior of a tumor was against the rule, its transplantation taking place more readily to hosts of an alien variety. Such instances are so rare as to deserve special report.

The growth in question, a tumor of the fowl (Chicken Tumor XVIII), has already been described at length.[1] It is a spindle-celled sarcoma curiously rifted with blood sinuses, and showing a tendency to metastasize to the voluntary muscles. It originated in a brown Leghorn hen, and was transferred to two successive series of such hosts, in which it grew slowly but with some increase in the percentage of takes. Of the third series of fowls inoculated several were barred Plymouth Rocks, and in these the tumor grew with especial rapidity. On subsequent inoculation to other barred Plymouth Rocks its growth was still more rapid. The findings seemed attributable either to an enhanced malignancy consequent on passage and independent of the host's variety, or to some special susceptibility of the Plymouth Rock breed. To test the matter a number of comparative inoculations were made.

* Received for publication, August 1, 1914.

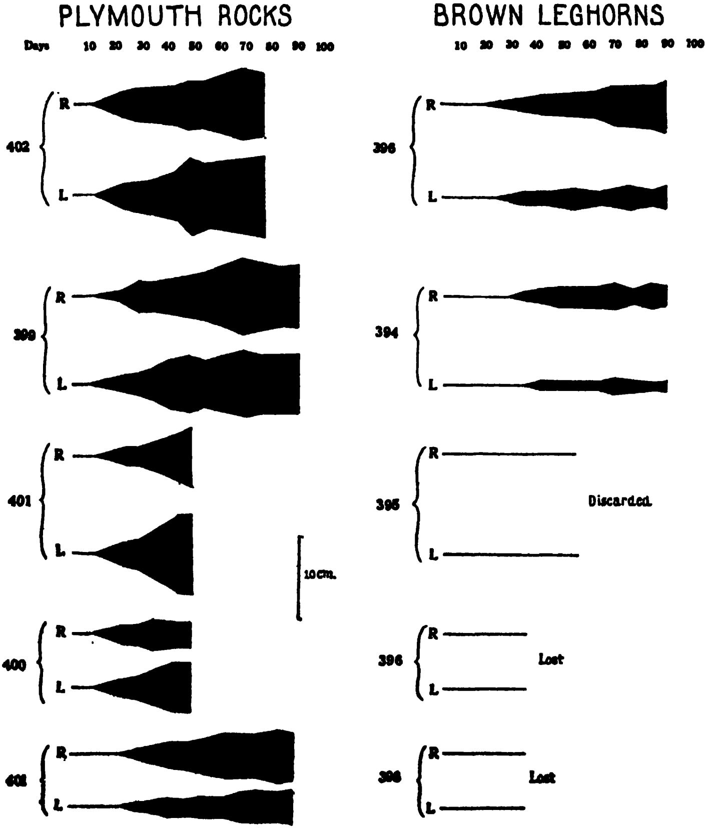
[1] Rous, P., and Lange, L. B., *Jour. Exper. Med.*, 1913, xviii, 651.



TEXT-FIG. 1. Results of the inoculation into Plymouth Rock and brown Leghorn fowls of bits of the rifted sarcoma taken from a Plymouth Rock. Two inoculations were made into each fowl. The time of appearance and rate of growth of the tumors are shown by diagrammatic outlines of which the width indicates the diameter of the tumor, and the length the period of growth. A hatched outline indicates that the tumor had filled the whole breast and could not be accurately measured. R and L = tumors of right and left breast. K = killed. The changes in size of the tumors appear abrupt, but this is because the measurements were taken at considerable intervals.

Experiment 1.—Small bits of fresh tumor tissue, of approximately equal size, from a barred Plymouth Rock fowl were implanted through a trocar, one in each breast of six brown Leghorn adults and six Plymouth Rocks. The results of the transplantation are shown in text-figure 1.

Experiment 2.—The tumor material was taken from a brown Leghorn inoculated in the preceding experiment. Implantations were made as before into each breast of five Plymouth Rock and five brown Leghorn fowls (text-figure 2).



TEXT-FIG. 2. Results of the inoculation into Plymouth Rock and brown Leghorn fowls of bits of the rifted sarcoma from a brown Leghorn fowl of the experiment recorded in text-figure 1.

Text-figure 1 shows that the sarcoma taken from a Plymouth Rock fowl and inoculated into Plymouth Rocks and brown Leghorns appeared in a smaller percentage of the latter and after a longer interval, and grew less well. This was also the case when the material was derived from a brown Leghorn fowl (text-figure 2). Further observations have confirmed these findings. It may be urged that in the experiments figured, the results were due to an adaptive change in the tumor consequent upon its growth in several series of Plymouth Rocks, and not to be altered by sojourn in a single series of brown Leghorns. But as already mentioned, results entirely similar were obtained with the first Plymouth Rock fowls inoculated.

There are certain obvious physical differences between brown Leghorn and Plymouth Rock chickens to which the results might, conceivably, be due. The one breed is small and wiry, whereas the other is large and fat. Other things being equal, one might suppose that as hosts for all sorts of proliferating tissue the Plymouth Rocks would be better. To throw light on this point fowls of both varieties were inoculated with Chicken Tumor I, a simple spindle-celled sarcoma. This growth has already been mentioned for the striking specificity whereby its successful transplantation was at first confined to blood related Plymouth Rocks. It is now very malignant and grows well in chickens of many breeds.

Experiment 3.—Inoculations of Chicken Tumor I from a Plymouth Rock hen were made, as in experiments 1 and 2, into each breast of five Plymouth Rock and five brown Leghorn fowls. The results are given in text-figure 3.

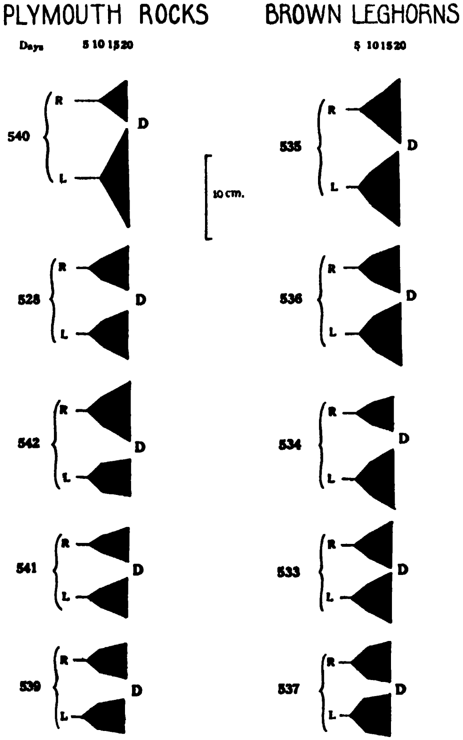
It will be seen (text-figure 3) that the tumor succeeded equally well in both varieties of fowls,—and this despite its early predilection for Plymouth Rocks. The findings with the rifted sarcoma can hardly be attributed then to gross physical differences in the chickens used.

Like our other transplantable chicken tumors the rifted sarcoma has a filterable agent as its cause. Yet in its transfer to new hosts, as carried out by introducing a bit of the fresh tumor tissue, a genuine transplantation is ordinarily involved, the new tumor arising by the survival and proliferation of the implanted fragment. The marked success of the sarcoma in hosts of an alien variety might conceivably be due either to an influence exerted on the transplanted cells or directly on the agent causing the disease. The results of a filtration experiment furnish some evidence for the latter view.[2]

[2] Rous, P., and Murphy, Jas. B., *Jour. Exper. Med.*, 1914, xix, 52.

Experiment 4.—Four Plymouth Rock fowls and four brown Leghorns were inoculated with equal portions of a Berkefeld filtrate prepared from the fresh tissue of the rifted sarcoma. A little sterile diatomaceous earth was added to the filtrate prior to its injection. The tumor used came from a Plymouth Rock fowl to which it had been transplanted after growing in two series of brown Leghorns.

The inoculated fowls were kept several months under observation. Two of the four Plymouth Rocks developed growths from which they eventually died. Of the four brown Leghorns only one developed a growth and this retrogressed.



TEXT-FIG. 3. Results of the inoculation of bits of the simple spindle-celled sarcoma into Plymouth Rock and brown Leghorn fowls. The tumor material was obtained from a Plymouth Rock.

No further experiments of the sort have been made because of the difficulty of obtaining the causative agent of the rifted sarcoma in active form. Unlike the agents causing our other chicken tumors it does not survive in the dried or glycerinated tumor tissue. Moreover, its activity in filtrates is very inconstant, and at best tumors are not produced until several months after the injection.

Among the numerous instances of the influence of race on the transfer of mammalian tumors we have been able to find but one in any way parallel to that here recorded. Tyzzer[8] transplanted a tumor of the Japanese mouse to hybrids of this breed and the ordinary white mouse, a variety completely insusceptible to the growth. In the F_1 generation of hybrids the tumor succeeded much better than in the Japanese mice. It may perhaps be remarked in this connection that Plymouth Rock fowls represent a mixture of several strains.

SUMMARY.

A transplantable sarcoma of the fowl, known as Chicken Tumor XVIII, in our series, succeeds better in chickens of an alien breed (Plymouth Rock) than in those of the variety in which it originated (brown Leghorn). This is not due to gross physical differences in the two breeds but to some more subtle factor and one which perhaps acts by influencing the agent causing the tumor. It would seem that Chicken Tumor XVIII, as it occurred in nature, was an instance of a disease appearing spontaneously in an animal of relatively insusceptible variety.

[8] Tyzzer, E. E., *Jour. Med. Research,* 1909, xxi. 519.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, October 1, 1914, Vol. xx, No. 4, pp. 419–432.]

ON IMMUNITY TO TRANSPLANTABLE CHICKEN TUMORS.*

BY PEYTON ROUS, M.D., AND JAMES B. MURPHY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The observation that the transplanted tumors of mice sometimes retrogress has been provocative of much research; for in it the phenomenon of acquired resistance to neoplasms was first clearly recognized. Now we know that this resistance is not peculiar to tumors but is elicited by non-neoplastic tissues as well. A resistance attributable to a causative element in mammalian new growths has still to be demonstrated, as, indeed, has such an element. Causative agents for transplantable chicken tumors, on the other hand, have been found. Those thus far studied are filterable. In the light of this fact a comparison of the phenomena of resistance to chicken tumors and resistance to mammalian growths becomes of much interest; since it may well be that there exist gross differences that would prove the two of different etiology. The present paper is concerned with such a comparison. In addition there will be taken up the question of the relationship between the agents causing different chicken tumors as indicated by the specificity of the resistance to them.

We have used for the work three distinct chicken tumors, namely, a simple, spindle-celled sarcoma (Chicken Tumor I), an osteochondrosarcoma (Chicken Tumor VII), and a spindle-celled sarcoma curiously fissured with blood sinuses and showing a tendency to metastasize to the skeletal muscles (Chicken Tumor XVIII). Most of the data have been obtained with Chicken Tumor I, which has been longest in our hands.

NATURAL RESISTANCE.

Natural resistance to the avian tumors will be briefly dealt with, since it has already been reported upon in describing the growths.

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* Received for publication, August 1, 1914.

Rat and mouse tumors, like the non-neoplastic tissues, can be successfully transferred under ordinary circumstances only to animals of the same species. This is true of chicken tumors as well. They will not grow in rats, mice, rabbits, or pigeons; and the spindle-celled sarcoma, the only one thus tested, will not grow in ducks. In fowls that are sick or emaciated the tumors do badly, either failing to develop after the inoculation, growing slowly, or retrogressing early. The same peculiarity has excited much attention in the case of mammalian growths. These latter grow best in young animals, and especially well in the new-born.[1] The influence of the age of the host upon chicken tumors has been tested only with the simple spindle-celled sarcoma. Young fowls have been found most susceptible as hosts for it, and in chick embryos it grows with extraordinary rapidity.[2]

Not a few mouse tumors are transplantable solely to animals of the variety in which the growth was spontaneous. A still greater specificity has been shown by Chicken Tumor I, which was transplantable at first only to blood relations of the original host and not to other varieties than the original until after months of propagation. The osteochondrosarcoma exhibits no preference for a special variety of fowl. The sarcoma rifted with blood sinuses shows what may be termed a reversed specificity, growing better in fowls of an alien sort (barred Plymouth Rock) than in the original brown Leghorn variety. This finding has been made the subject of a special paper.[3]

There exists an individual resistance to mammalian growths independent of all the factors thus far mentioned. Animals possessing it in its complete form fail to develop a tumor even though inoculated again and again. This is true of chicken tumors as well. But it is noteworthy in both cases that as the malignancy of the growth increases, owing to its sojourn in susceptible hosts, the number of animals insusceptible to it lessens. An individual naturally resistant to one form of mammalian tumor is frequently very susceptible to another. There is abundant evidence that this is true of avian growths as well.

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[1] Unpublished work from this laboratory.

[2] Rous, P., and Murphy, Jas. B., *Jour. Am. Med. Assn.*, 1911, lvi, 741.

[3] Rous, P., and Lange, L. B., *Jour. Exper. Med.*, 1914, xx, 413.

Experiment 1.—Nine fowls were employed, four of them normal Plymouth Rocks, and the remainder brown Leghorns naturally resistant to the rifted sarcoma, as is shown by its failure to develop in them on a previous inoculation. All were now inoculated in the muscle of one leg with a bit of a slowly growing simple sarcoma (Chicken Tumor I), in the other with the rifted sarcoma. The inoculation of the simple sarcoma was unsuccessful and after seventeen days a second inoculation was made at the same site with more malignant material. The rifted sarcoma was then just beginning to appear. The final results are shown in text-figure 1.

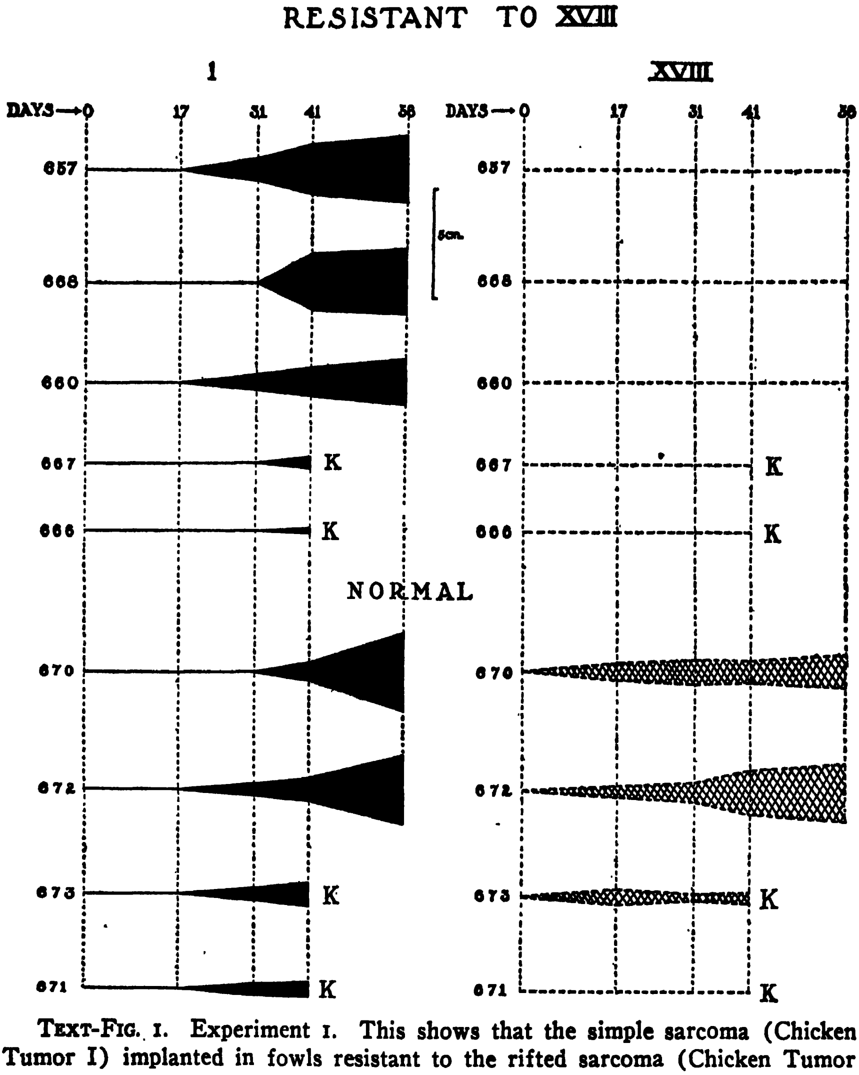
Text-figure 1 shows that fowls with a complete natural resistance to the rifted sarcoma and perhaps a slight acquired one (from the previous inoculation) were as susceptible to the simple sarcoma as normal fowls in which the rifted sarcoma grew well.

From all of the foregoing it is plain that the phenomena of natural resistance to chicken tumors are, in general, strikingly similar to those associated with rat and mouse tumors. The only apparent exception is in the tendency shown by the rifted sarcoma to grow better in fowls of an alien variety. Even here an instance somewhat similar may be found in mammals. Tyzzer bred together mice of two varieties, the one susceptible, the other insusceptible to a transplantable tumor of the Japanese waltzing mouse and found that the offspring of the F_1 generation were more susceptible than the susceptible parent. In the case of the rifted sarcoma the varieties of host tested were both the result of interbreeding several strains of fowls.

ACQUIRED RESISTANCE.

Some kinds of transplantable mammalian tumors grow progressively until the death of the animal; others after brief growth tend to become stationary and retrogress. The osteochondrosarcoma's behavior is of this latter sort. After a period of rapid enlargement as a chondrosarcoma in which spicules of bone gradually appear, it in most cases ceases to grow and is slowly absorbed. It not infrequently retrogresses after reaching a diameter of six or seven centimeters, but may take months to disappear, especially when it contains much bone. Only by the careful selection of tumors still growing has it been propagated. The simple spindle-celled sarcoma as a rule develops rapidly and progressively; but by the transplantation of slowly growing examples a retrogressing form may be obtained.

The rifted sarcoma develops slowly and with a considerable proportion of retrogressions.



Text-Fig. 1. Experiment 1. This shows that the simple sarcoma (Chicken Tumor I) implanted in fowls resistant to the rifted sarcoma (Chicken Tumor XVIII) grew as well as in normal fowls susceptible to the latter.

The time of appearance and rate of development of each tumor are shown by diagrams of which the width represents the diameter of the growth and the length its period of existence. A straight line indicates that no tumor developed. Cross-hatching indicates a rifted sarcoma, and solid black a simple sarcoma. The two are grouped in separate columns. The fowls are Nos. 657, 668, etc. K = killed.

The amount of tumor material implanted has a marked influence on the course of all three chicken tumors. Retrogressing growths follow much more frequently the inoculation of single, small tumor bits than they do the inoculation of one to two cubic centimeters of the same tumor tissue, ground to a pulp. That dosage has an influence on the development and course of mammalian growths has long been known (Loeb, Clowes, and Baeslack).

Rats and mice in which tumors retrogress acquire resistance, as is shown by the fact that reinoculations within a few weeks usually fail of success. Some mouse tumors confer resistance on the host while they are still growing. This is especially true of tumors of retrogressing tendency. In the case of certain other neoplasms the factors which determine the course of the disease are so balanced that by mechanical means a stationary tumor may be made a growing one;[4] or reinoculations into a host in which the growth is stationary or retrogressing may be successful.

Exactly the same phenomena have been noted of the chicken tumors. The spindle-celled sarcoma grows rapidly and the success of secondary inoculations shows that it produces no notable concomitant resistance. The few individuals in which it is absorbed are usually resistant for a considerable time. The osteochondrosarcoma, a growth which tends to retrogress, produces a strong concomitant resistance (text-figures 2 and 5). Chickens in which it has been present for several weeks are always absolutely resistant on secondary implantation, and this at a period when the primary tumor is still growing. The slowly developing, rifted sarcoma often becomes stationary for long periods and then starts to grow again. By the use of malignant material hosts in which this growth is stationary or even slowly disappearing may sometimes be successfully reinoculated.

The resistance induced by the retrogression of a rat or mouse tumor is in part a pan-resistance but is most effectual against tumors of the same sort. Whether a pan-resistance to chicken tumors follows their retrogression has not been determined, but certainly much of the resistance is specific, as the following experiment shows.

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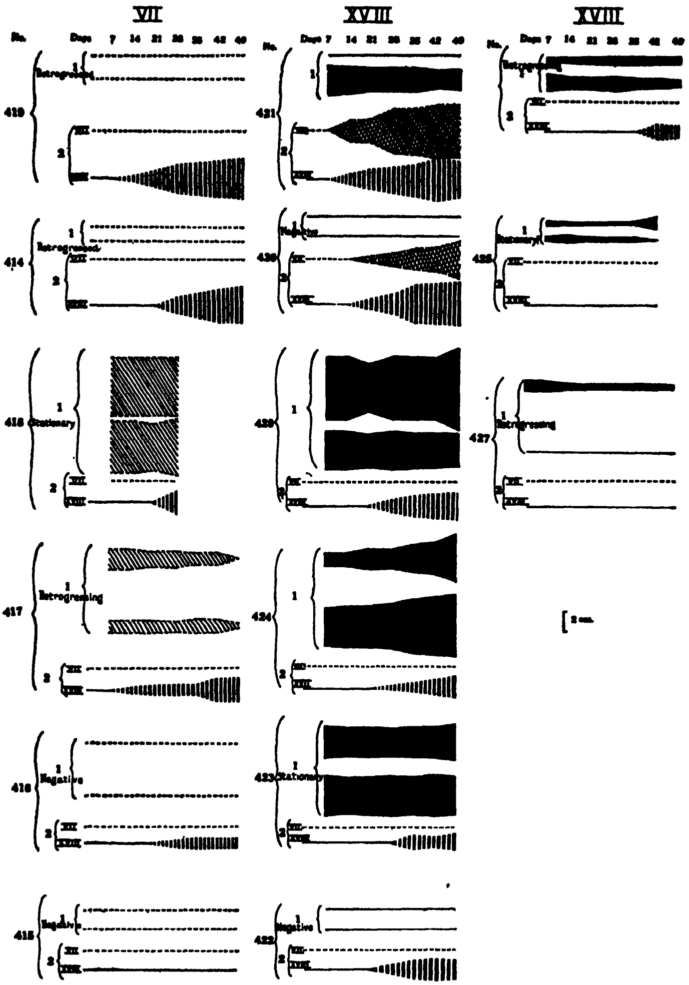
[4] Loeb, L., *Jour. Med. Research*, 1901, vi, 28.



TEXT-FIG. 2. Experiment 2. This text-figure illustrates the fact that acquired resistance to the rifted sarcoma (Chicken Tumor XVIII) is slight as compared with that to the osteochondrosarcoma (Chicken Tumor VII); and it shows furthermore that resistance to the latter growth is to a large extent specific. All of the fowls (Nos. 419, 414, etc.) had been inoculated previously at two points. The character of this first inoculation (Chicken Tumor VII or Chicken Tumor XVIII) has determined the grouping in columns. For each fowl there are four diagrams representing tumor growth, or, in its absence, four lines. The diagrams bracketed together as 1 record the tumors of first inoculation; those bracketed as 2 record those of the second, the latter comprising an implantation with both Chicken Tumors VII and XVIII. The diagrams of the rifted sarcomata (Chicken Tumor XVIII) of first inoculation are given in solid black, those of the second in heavy hatching. A lightly hatched diagram indicates an osteochondrosarcoma of the first inoculation, and a cross-hatched one a tumor of the same sort following the second inoculation.

Experiment 2.—Six fowls previously inoculated with the osteochondrosarcoma and nine inoculated with the rifted sarcoma were chosen for this experiment. Some carried growths that were enlarging, some retrogressing growths, and others had shown themselves naturally resistant. All were now inoculated with the rifted sarcoma in the wing muscles of one side and with the osteochondrosarcoma at the same spot on the other side. 0.1 c.c. of a suspension of the fresh tumor tissue in Ringer's solution was used in each case. The course of the old tumors and the development of the new are shown in text-figure 2.

It will be seen from text-figure 2 that all of the fowls previously inoculated with the osteochondrosarcoma were now resistant to it. The malignancy of the material employed is proved by the rapidity with which it gave rise to tumors in two fowls previously implanted with the rifted sarcoma. This latter tumor grew in all but one of the fowls resistant to the osteochondrosarcoma. It also grew in seven of the nine hosts previously inoculated with a growth of its own sort. In one fowl the tumor of the first inoculation was actually retrogressing while that of the second enlarged.

When implanted simultaneously in the same host the chicken tumors preserve their character unchanged. The simple sarcoma metastasizes, as usual, to the lungs and other viscera, and the rifted sarcoma still gives secondary growths in the muscles, the source of each dissemination being clearly traceable from its histology. Sometimes one tumor grows rapidly whereas the others do badly or fail to grow (text-figure 4). So too it is with neoplasms of the rat and mouse. In a previous article the fact has been pointed out that the histological signs of resistance to these latter are identical with those to chicken tumors when allowance is made for the peculiarities of the two classes of host.[5]

Despite the efforts of many workers an immune principle effective against rat and mouse tumors has yet to be demonstrated in the blood of animals recovered from these growths. Crile and Beebe[6] succeeded in curing dogs of infectious lymphosarcoma by transfusing to them blood from other dogs in which the growth had retrogressed; but the lymphosarcoma has characters which distinguish it from the true neoplasms. Nevertheless, attempts to cure chicken tumors by means of transfusion have seemed advisable. Five fowls

[5] Rous, P., and Murphy, Jas. B., *Jour. Exper. Med.*, 1912, xv, 270.

[6] Crile, G. W., and Beebe, S. P., *Jour. Med. Research*, 1908, xviii, 385.

in which a relatively non-malignant form of the simple sarcoma was developing as the result of inoculation were bled from thirty-five to sixty-five cubic centimeters and an equal or slightly larger amount of blood was transfused to them from resistant fowls. In these latter the simple sarcoma had retrogressed and several intraperitoneal inoculations of sarcomatous tissue had from time to time been made without yielding tumors, a fact confirmed at autopsy. Transfusion was done at a time when resistance to the sarcoma may be supposed to have been at its greatest, that is to say, some two to three weeks after a massive injection of sarcomatous tissue. But in the fowls receiving the blood the tumors grew quite as well as in untransfused controls.

It is well known that not only does the retrogression of a mammalian tumor render the host unfavorable for subsequent tumor grafts but that injections of normal tissues, of normal blood even, will act to this end. Embryonal tissue is especially effective. In our experience the injection of hashed chick embryo does not confer resistance to the spindle-celled sarcoma of the fowl. But the tumor used was very malignant and may not have been sufficiently sensitive as an indicator.

Thus far the chicken tumors have been considered simply as transplantable new growths. The phenomena of acquired resistance to them resemble such as are seen under like conditions in the case of mammalian growths and suggest no more than these the presence of a causative agent distinct from the tumor cells.

RESISTANCE TO THE TUMOR-PRODUCING AGENTS.

By a special method there have been demonstrated two distinct forms of resistance against the simple sarcoma when it is transferred by grafting,—the one directed against the transplanted tumor cells, the other against the growth's causative agent.[7] Resistance of the latter sort will come into consideration in the findings now to be discussed.

With the exception of Königsfeld[8] workers with mammalian tumors have found that neoplastic tissue killed by drying fails to induce resistance against subsequent grafts. We have repeatedly attempted to induce with dried tissue resistance to the spindle-celled sarcoma of the fowl. The growth's causative agent remains active after drying, so it was necessary to make the first inoculations with material rid in some way of its tumor-producing property. The dried and powdered tissue was taken up in distilled water, heated at 60° C. for fifteen minutes, and injected intraperitoneally. For the later injections material submitted to 55°, 53°, or 50° C. for fifteen minutes, and finally unheated material, was used. Several groups of fowls were employed, but few came to the eventual test with the implanted growth, because nearly all developed tumors following the inoculation with unheated, dried tissue. Those remaining may well have been naturally resistant. If any protection is elicited by the injection of dried material in which the agent exists in attenuated form, it must certainly be very slight.

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[7] Rous, P., *Jour. Exper. Med.*, 1913, xviii, 416.

[8] Königsfeld, H., *Centralbl. f. Bakteriol.*, *1te Abt.*, *Orig.*, 1914, lxxiii, 316.

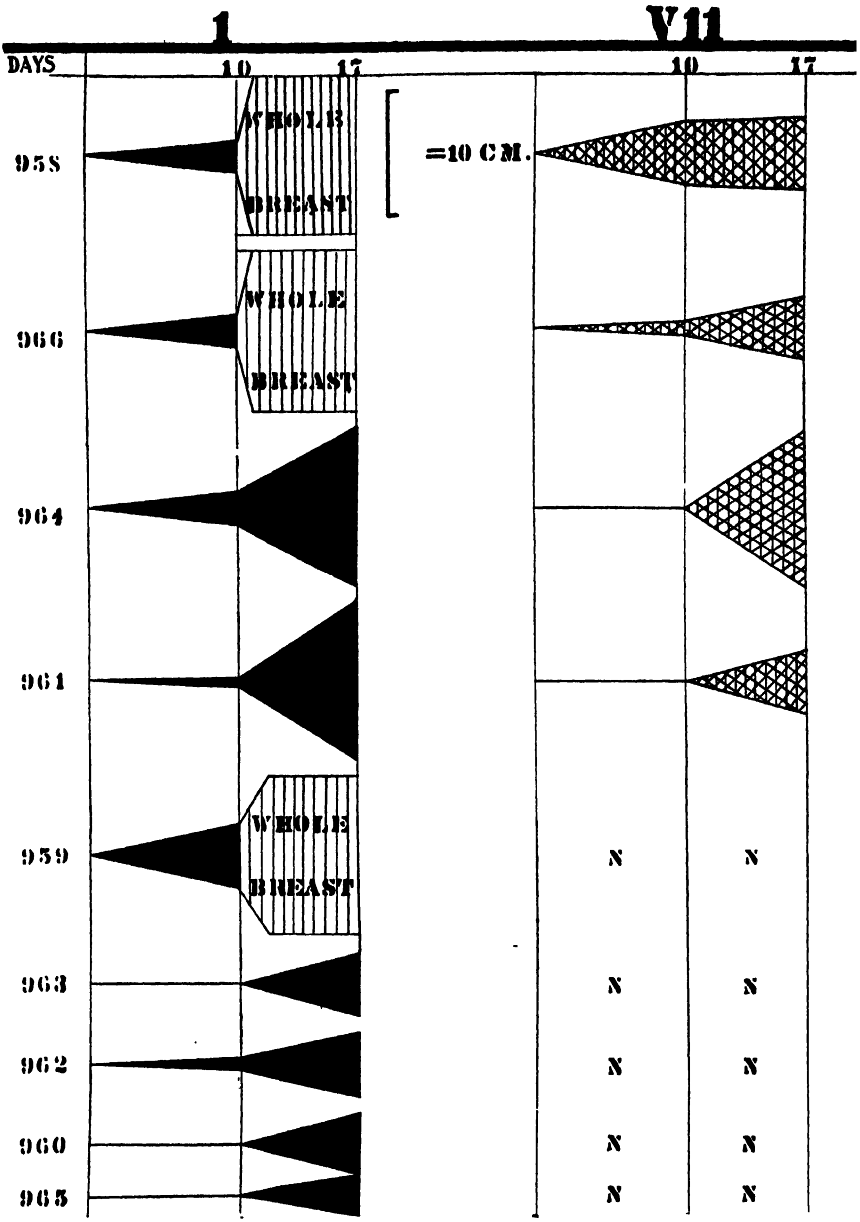
SPECIFICITY OF THE RESISTANCE.

That there exists a natural resistance to the agents is shown by their failure to produce tumors in some hosts. The question arises as to how far this resistance is specific.

Experiment 3.—Nine healthy Plymouth Rock fowls were inoculated, in one breast with 0.5 c.c. of a suspension of the dried tissue of an osteochondrosarcoma (Chicken Tumor VII), in the other breast with 0.1 c.c. of a like suspension of the dried spindle-celled sarcoma (Chicken Tumor I). The suspensions were made by rubbing up 1 gm. of dried tumor tissue in 9 c.c. of distilled water. The difference in dosage was to compensate for differences in the malignancy of the tumors. The results will be found in text-figure 3.

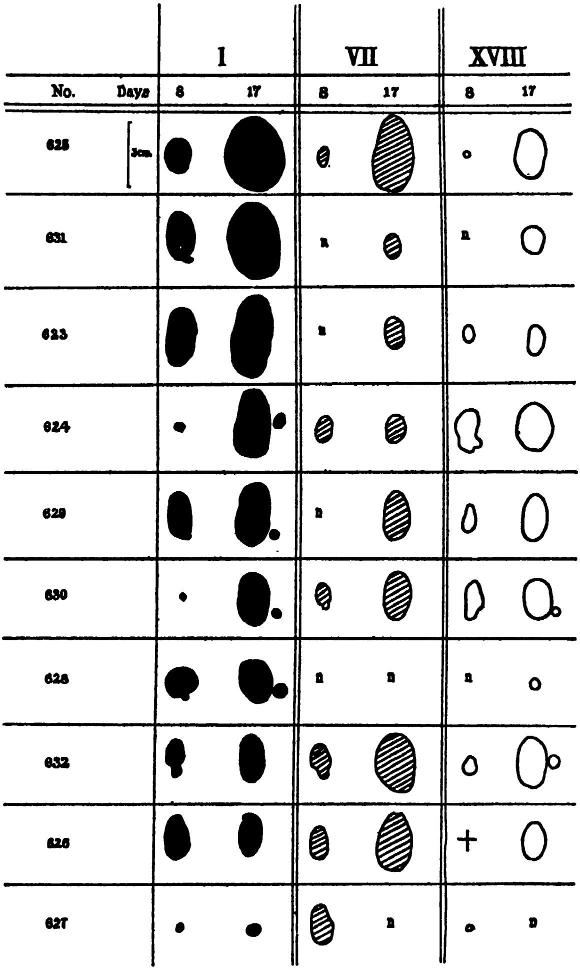
It is evident from text-figure 3 that the agents of the simple sarcoma and the osteochondrosarcoma are largely influenced by the same factors of natural resistance. In the experiment which it illustrates the period which elapsed before the appearance of a palpable tumor was so nearly the same for the two growths that the results can scarcely be referred to concomitant resistance induced by one tumor and effectual on the other.

With the rifted sarcoma a test of the above sort has not been possible because its agent is obtained apart from living cells only inconstantly and with difficulty. Comparative transplantation has been resorted to but this introduces a factor of error in that there



TEXT-FIG. 3. This shows that the same factors of natural resistance influence the activity of the agents causing two different chicken tumors. The fowls (Nos. 958, etc.) were inoculated in one breast with dried material of the simple sarcoma (Chicken Tumor I), in the other with that of the osteochondrosarcoma (Chicken Tumor VII). The diagrams are black for the simple sarcoma, crosshatched for the osteochondrosarcoma.

are transferred with the agent tumor cells strange to the new hosts yet capable of active proliferation in many of them. With such a large disturbing element one would scarcely expect to learn much regarding the specificity of resistance to the agents. The following experiment gives evidence for the correctness of this view.

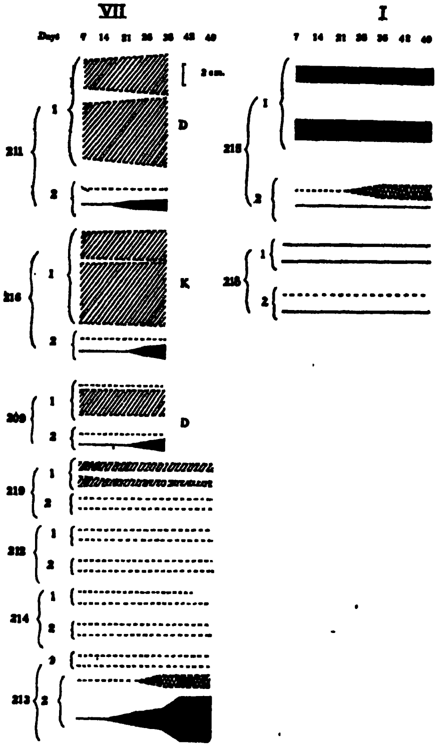


TEXT-FIG. 4. Fowls Nos. 625, etc., were inoculated simultaneously at different points with all three chicken tumors (I, VII, and XVIII). The text-figure shows that the growths varied independently of one another.

Experiment 4.—Ten healthy Plymouth Rock fowls were inoculated at different spots with all three chicken tumors (I, VII, and XVIII) in the amount of

0.1 c.c. of the finely ground fresh tissue. The sites chosen were in the muscle of both breasts, and the arrangement of the inoculations was varied from fowl to fowl. The results are shown in text-figure 4.

It will be seen from text-figure 4 that the tumors varied independently of one another. The findings as regards the simple sarcoma and the osteochondrosarcoma give no hint of the relationship seen in text-figure 3.



Text-Fig. 5. This text-figure has to do, like text-figure 2, with reinoculations; and the same general explanation holds good for it. The results of the first inoculation are given in the bracket 1 and those of the second in 2. The black and hatched diagrams are those of the simple sarcoma and the osteochondrosarcoma, respectively. The second inoculation was made with dried material of each growth. It will be seen that the agent of the simple sarcoma failed to give rise to tumors in fowls in which this growth had done badly on previous inoculation, whereas it caused growths in fowls resistant to the osteochondrosarcoma. The resistance against the latter tumor growth is also largely specific. Of seven fowls previously inoculated with it but one was susceptible on second inoculation. This fowl, No. 213, was supposed to be naturally resistant because of an unsuccessful inoculation some weeks previously, but the agents of both tumors engendered growths in it.

SPECIFICITY OF ACQUIRED RESISTANCE TO THE AGENTS.

Obviously the resistance acquired by a fowl in which a tumor has retrogressed must be effectual not only against the tumor cells but against the associated agent,—else this latter by acting on the cells of the host would produce a tumor. The following experiment indicates that acquired resistance to a tumor-producing agent is largely specific.

Experiment 5.—One fowl in which the simple sarcoma had retrogressed, one in which it was stationary, and four fowls carrying the osteochondrosarcoma were employed. They were inoculated, in one breast with 0.1 c.c. of a thin paste made by rubbing up dried tissue of the simple sarcoma with Ringer's solution, in the other with 0.5 c.c. of a similar paste of the dried osteochondrosarcoma.

As text-figure 5 shows, the four fowls carrying the osteochondrosarcoma evinced a complete resistance to it on secondary inoculation, whereas the simple sarcoma developed in three of them. The opposite result was obtained with the fowls in which the simple sarcoma had retrogressed or was stationary. Both now proved resistant to this growth, but in one the osteochondrosarcoma developed. The fact that the agent of the osteochondrosarcoma is relatively inactive renders the result more striking.

SUMMARY.

The phenomena of natural and acquired resistance to transplanted chicken tumors strikingly resemble those observed in the case of transplanted mammalian growths; and no more than those do they suggest that the tumors have an extrinsic cause.

That there may exist in fowls implanted with a chicken tumor a resistance directed against the tumor-causing agent distinct from the resistance manifested against the alien tumor cells has been shown in a previous article.[9] Both sorts of resistance are present in a fowl in which a tumor has retrogressed, the resistance in such an instance being acquired. That directed against the agent is largely specific, giving little if any protection against the agents

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[9] Rous, P., *loc. cit.*

causing other tumors. There is some evidence that the conditions upon which a fowl's natural resistance depends are the same for the agents causing different chicken tumors.

It has proved impossible to protect chickens against the agent causing the simple sarcoma by injecting them with dried tumor material in which this agent has been attenuated by heat. The transfer of blood from resistant fowls to fowls with growing tumors is in our experience void of effect on the tumors.

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STUDIES IN CARBOHYDRATE METABOLISM.

III. THE INFLUENCE OF HYDRAZINE UPON GLYCOGEN STORAGE IN THE ORGANISM, AND UPON BLOOD COMPOSITION.*[1]

BY FRANK P. UNDERHILL.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

In a previous communication[2] it has been demonstrated that suitable quantities of hydrazine salts subcutaneously administered to dogs cause a marked hypoglycaemia with an accompanying reduction in the glycogen content of the liver. An explanation for this behavior is lacking. The present investigation was planned to determine whether the diminution of sugar in the blood and the disappearance of glycogen from the liver is coincident with a corresponding increase of glycogen in the muscles. Such a transformation of carbohydrate material is an obvious possibility and before seeking a more elaborate interpretation of the phenomenon under discussion it seemed desirable to exclude the possibility mentioned. Also in order to detect some of the more apparent alterations in blood composition that might account for decreased sugar content, the solids and ash content of the blood of hydrazinized and normal dogs subjected to similar experimental conditions have been compared.

In all instances the animals had been kept upon a mixed diet previous to the experiment and they were in splendid nutritive condition. The hydrazinized dogs were killed forty-eight hours subsequent to the injection and since these animals refused food during this length of time the dogs receiving no hydrazine were allowed to fast two days also. The sugar content of the blood and the glycogen content of the liver and muscles were determined by the respective methods indicated in a previous paper.[8] The solid and ash contents were estimated by the well known procedures.

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* Received for publication, January 30, 1914.

[1] Aided by a grant from The Rockefeller Institute for Medical Research.

[2] Underhill: this *Journal*, x, p. 159, 1911.

A Comparison of the Composition of Blood and of the Content of Glycogen in Liver and Muscle in Hydrazinized and Normal Dogs.

NUMBER OF DOG	SUBCUTANEOUS INJECTION OF HYDRAZINE SULPHATE	BLOOD			GLYCOGEN CONTENT OF LIVER EXPRESSED AS GRAMS OF DEXTROSE	GLYCOGEN CONTENT OF MUSCLES (LEG) EXPRESSED IN PERCENTAGES OF DEXTROSE
		Sugar Content	Solids	Ash		
	mgm. per kilo	*per cent*	*per cent*	*per cent*		
2	50	0.04	24.93	0.72	trace	0.75
9	50	0.06	26.61	0.32	0	0.44
10	50	0.04	25.25	0.33	0	0
12	50	0.05	26.00	0.84	0	0.70
6	0	0.14	16.50	0.87	12.7	0.92
7	0	0.19	19.60	0.87	6.5	1.50

An inspection of the table presented will demonstrate clearly that a transference of the body carbohydrate, at least as glycogen, to the muscles will not suffice as an explanation for the disappearance of glycogen from the liver and the decreased blood-sugar content after the subcutaneous introduction of hydrazine sulphate to dogs. On the other hand, there are indications not only that the liver is depleted of its glycogen store, but that the amount of muscle glycogen may be markedly decreased and indeed at times may disappear entirely thus presenting an animal with a minimum of carbohydrate in its body. The data compare well with what may be obtained at times with phlorhizin except that in the case of the hydrazinized animal sugar never appears in the urine. It is also of interest to note that the solids of the blood may be abnormally high although the ash content may be distinctly subnormal. Although the number of experiments is perhaps too small to admit a positive statement it is worthy of note at least that there is more or less parallelism between the ash content of the hydrazinized dogs' blood and the percentage of glycogen in the muscles.

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[8] Underhill: *loc. cit.*

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, March, 1914, Vol. xvii, No. 2, pp. 295-298.]

STUDIES IN CARBOHYDRATE METABOLISM.

IV. DO HYDRAZINE DERIVATIVES SHOW THE TYPICAL HYDRAZINE EFFECT UPON BLOOD SUGAR CONTENT?*[1]

BY FRANK P. UNDERHILL.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

The influence of hydrazine upon carbohydrate metabolism leading as it may to either the prevention or the inhibition of pancreatic diabetes[2] suggested the possibility of finding some derivative of the compound mentioned which while still exhibiting the effect upon blood sugar content would nevertheless be free from a part, at least, of the great toxicity of hydrazine. In accordance with this idea a number of hydrazine derivatives were procured, but it was soon found that only a few could be employed for the purpose outlined above owing to the great insolubility of the substances. Experiments have been carried through, however, with the following compounds, methylhydrazine, phenylhydrazine, methylphenylhydrazine, diphenylhydrazine, and semicarbazide.

It may be stated at once that in no instance was there the influence upon carbohydrate metabolism of dogs that is so peculiarly associated with hydrazine itself.

EXPERIMENTAL.

The dosage employed was similar to that for hydrazine and in all cases the dogs starved subsequent to the administration of the drug. Since hydrazine produces its maximum effect in about forty-eight hours the blood sugar estimations in the present investigation were made after a similar lapse of time. The relative toxicity of

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* Received for publication, January 30, 1914.

[1] Aided by a grant from The Rockefeller Institute for Medical Research.

[2] Underhill and Fine: this *Journal*, x, p. 271, 1911.

the investigated substances was determined in rabbits. When it had been shown that a dose similar to the usual hydrazine dosage, *i. e.,* 50 mgm. per kilo, could be borne, an experiment was carried out with the dog. All injections were made subcutaneously and the behavior of the animal closely watched.

METHYLHYDRAZINE, $NH_2.NH.CH_3$.

Action upon Rabbit.—At 11.00 a.m. 0.095 gram of methylhydrazine (Schuchart) was injected into a rabbit of 1900 grams (50 mgm. per kilo). No unusual symptoms were observed until 2.45 p.m. at which time the animal urinated and immediately was seized with convulsions—episthotonus—the fore legs appearing to be paralyzed. The convulsion lasted only two or three minutes, after which the rabbit lay sprawled on its belly.

3.00 p.m.	Second convulsion.
3.15	Third convulsion. Hind legs seem paralyzed also.
3.20	Fourth convulsion.
3.45	Fifth convulsion. Grinds teeth. In constant tremor, makes sudden jumps, fore legs jerk spasmodically and continually. Salivation, mouth open, air hunger (?).
3.53	Convulsion. Urination.
3.55	Convulsion. Animal lies prostrate.
3.57	Convulsion.
4.10	Rabbit in constant motion—as though jumping at something.

Convulsions and constant motions continued up to 6.00 p.m. Animal was found dead the next morning. Autopsy showed nothing abnormal.

Urine voided after injection of methylhydrazine reduced Benedict's solution even in the cold solution. Since methylhydrazine shows a similar behavior it is probable that this substance or a closely related compound was rapidly eliminated by the kidneys.

A second rabbit upon receiving an injection of 25 milligrams of methylhydrazine per kilo gave no evidence of any symptoms described for the larger dose.

Action upon the Dog.—The subcutaneous injection of 0.42 gram (35 mgm. per kilo) methylhydrazine into a 12-kilo dog caused the animal to vomit and to refuse all food. Normally the dog was very active but after the injection she became very sluggish and lay quietly in the cage. No other symptoms developed. At the end of two days blood was drawn from the carotid and *the blood sugar content was found to be 0.11 per cent.* Glycogen was present in the liver to the extent of 0.25 gram expressed as dextrose. The liver did not present the appearance characteristic of hydrazine. The other principal organs seemed normal.

PHENYLHYDRAZINE, $NH_2.NHC_6H_5$.

Action upon the Dog.—A dog of 10 kilos was given an injection of 50 mgm. per kilo of phenylhydrazine hydrochloride without producing any unusual symptoms beyond the appearance of a relatively large amount of methaemoglobin in the urine. On the second day subsequent to the injection blood was drawn from the carotid and *was found to contain 0.16 per cent dextrose.* The glycogen content of the liver amounted to 0.20 gram expressed as dextrose. Upon autopsy all organs appeared normal except the liver and spleen. The former was of a peculiar chocolate brown color. The spleen was enormously enlarged and dark purple in color.

METHYLPHENYLHYDRAZINE, $NH_2.(CH_3)N.C_6H_5$.

Action upon the Rabbit.—The injection of 50 mgm. per kilo of methylphenylhydrazine sulphate into a rabbit of 1200 grams produced no abnormal symptoms.

When 100 mgm. per kilo of the same salt were introduced into a rabbit of 1600 grams no evidence of toxicity was observed.

Action upon the Dog.—A dog of 15 kilos received an injection of 1.5 grams (100 mgm. per kilo) of the above mentioned salt at 10.00 a. m. At 10.30 the animal lay prostrate and was unable to rise. The symptoms gradually became more and more pronounced until death which occurred at 12.30, apparently from respiratory failure. No autopsy was made.

To a second dog of 4 kilos was given an injection of the same salt in the dosage of 50 mgm. per kilo. Soon after the administration of this compound the dog vomited and gave evidence of muscular weakness. These symptoms gradually disappeared and in a few hours the animal seemed normal. Two days after the injection blood was collected from the carotid. *The blood sugar content was 0.14 per cent.* Glycogen in the liver amounted to 12.6 grams expressed as dextrose. All organs and tissues seemed normal.

DIPHENYLHYDRAZINE, $NH_2.N(C_6H_5)_2$.

Action upon the Rabbit.—The introduction of the above compound as the hydrochloride into rabbits failed to produce abnormal symptoms in doses of either 50 or 100 mgm. per kilo.

Action upon the Dog.—To a dog of 10 kilos was given an injection of the above mentioned hydrazine derivative (100 mgm. per kilo) without any subsequent sign of abnormality except that the animal lay quietly in the bottom of the cage. Two days later the dog seemed normal and was bled from the carotid. Sugar was *present in the blood to the extent of 0.19 per cent.* In the liver 6.4 grams of glycogen expressed as dextrose were found. All organs were normal.

SEMICARBAZIDE, $NH_2.NH.CO.NH_2$.

Action upon the Rabbit.—A rabbit of 1300 grams received an injection of 65 mgm. of semicarbazide hydrochloride (50 mgm. per kilo). For a few hours an undue excitation was observed which gradually passed away.

In doses of 100 mgm. per kilo this salt caused the death of a rabbit of 1500 grams within twenty-four hours, without evidence of any significant symptoms preceding.

Action upon the Dog.—To a dog of 10 kilos was given an injection of 0.5 gram of semicarbazide hydrochloride (50 mgm. per kilo). No abnormal symptoms followed. Two days later the blood sugar content amounted to 0.10 per cent. In the liver were found 4.5 grams of glycogen expressed as dextrose. All organs were normal.

[Reprinted from The Journal of Biological Chemistry, March, 1914, Vol. xvii, No. 2, pp. 299-304.]

STUDIES IN CARBOHYDRATE METABOLISM.

V. The Disappearance of Sugar from Solutions Perfused through the Heart of the Normal Rabbit, and of Animals Subjected to Inanition and to the Action of Hydrazine.*[1]

By FRANK P. UNDERHILL and A. L. PRINCE.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, and the Physiological Laboratory, Yale Medical School, New Haven.)

Among the many suggestive explanations for the greatly diminished sugar content of the blood,[2] and the almost (and at times quite), complete disappearance of glycogen from the liver and muscles[3] of hydrazinized animals, stands prominent the possible rôle of muscle tissue. It may be assumed, for example, that the activity of certain enzymes may be greatly accelerated by hydrazine leading to increased carbohydrate combustion or other transformation. Granting such an hypothesis it is reasonable to suppose that an acceleration of enzyme activity would be more or less commonly distributed to all active muscles. As an example of active muscle tissue the heart may be selected and a study of the disappearance of sugar from a solution perfused through this organ might be expected to throw some light upon the problem under discussion. The results of such a study form the basis of the present paper.

Methods.—The heart employed was that of the rabbit, it having been demonstrated[4] that hydrazine in sufficient quantities may produce the effects usually observed in dogs. The perfusions were made with Locke's solution and in a somewhat modified Locke's

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* Received for publication, January 30, 1914.

[1] Aided by a grant from The Rockefeller Institute for Medical Research.

[2] Underhill: this *Journal*, x, p. 159, 1911.

[3] Underhill: See previous paper.

[4] Unpublished experiments.

apparatus. Sugar estimations in the blood were carried through by the method of Forschbach and Severin;[5] in the perfusion fluid the Allihn procedure was followed. In initiating an experiment the animal was rendered unconscious by a blow on the neck, bled, and the heart quickly removed, washed as free as possible from blood in dishes of sugar-free Locke's solution and then attached to the apparatus. With the above mentioned sugar-free salt solution the heart was perfused until entirely free from blood. The apparatus was then emptied and Locke's solution, containing approximately 0.1 per cent dextrose, added. In all instances the heart beat rapidly and vigorously, the perfusion usually being rapid. As a rule the variation in heart beat was from 74 to 144 beats per minute and the drip varied accordingly. Unless otherwise stated all experiments lasted for a period of two hours. This period was selected in order to avoid any pronounced influence that could be attributed to bacterial contamination. To further rule out bacterial activity the entire apparatus was sterilized just before each experiment, the heart was isolated and attached under conditions as aseptic as possible and only sterilized wash and perfusion fluids were employed. After the period of perfusion the apparatus and heart were thoroughly perfused with sugar-free salt solution. The heart was weighed after being cut open, washed and dried with filter paper. Control trials with the apparatus alone demonstrated a loss of dextrose to the extent of approximately 2 mgm., an amount too small to play a rôle in the results to be considered below.

It has been determined that in general a maximum effect of hydrazine upon the blood sugar content is obtained in approximately two days. During this period the animals refuse all food. In our preliminary experiments to determine the disappearance of sugar from the perfusion fluid passed through non-hydrazinized hearts we chose as our controls animals subjected to inanition for a period of two days. The results of experiments with these rabbits served as our "normals" with which to compare those obtained with hydrazinized animals.

Experiments 1 to 11 inclusive, Table 1, show the figures obtained with our control rabbits, figures which correspond well with those of Locke[6] but much lower than those recorded by Wilenko.[7] Calculated upon the basis of per gram of heart weight it is evident that per hour from 0.7 mgm. to 1.6 mgm. of dextrose may disappear from the perfusion fluid.

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[5] Forschbach and Severin: *Arch. f. exp. Path. u. Pharm.*, lxviii, p. 341, 1912.

TABLE I.

The Disappearance of Sugar from a Dextrose Solution Perfused through the Beating and Non-Beating Rabbit's Heart.

NUMBER OF EXPERIMENT	WEIGHT OF HEART	TOTAL SUGAR DISAPPEARANCE	SUGAR DISAPPEARANCE PER GRAM HEART PER HOUR	REMARKS
	grams	*mgm.*	*mgm.*	
1	7.0	17.6	1.2	No food for 2 days.
2	6.2	23.2*	0.9	" " " 2 "
3	6.0	12.0†	0.7	" " " 2 "
8	6.0	12.0	1.0	" " " 2 "
11	5.0	16.0	1.6	" " " 2 "
44	3.6	10.5	1.4	" " " 2 "
33	5.2	40.2	3.8	" " " 3 "
31	4.3	18.0	2.0	" " " 4 "
34	4.6	26.7	2.9	" " " 4 "
35	4.8	21.5	2.2	" " " 6 "
32	4.4	25.8	2.9	" " " 6 "
38	5.9	26.0	2.2	" " " 6 "
36	6.2	27.9	2.2	" " " 8 "
39	3.7	17.8	2.3	" " " 8 "
24	5.3	13.0	1.3	Well fed animal from pen.
26	4.3	10.0	1.1	" " " " "
40	3.4	12.0	1.7	" " " " "
41	3.6	12.0	1.6	" " " " "
42	5.2	25.5	2.4	" " " " "
43	3.2	10.0	1.5	" " " " "
37	4.8	18.8	1.8	Well fed animal given 15 grams dextrose by mouth about 18 hours previous to experiment.

Non-Beating Heart.

45	5.5	12.0	1.0	Rabbit from pen.
48	3.8	19.0	2.4	Rabbit from pen.
46	3.8	15.0	2.1	Rabbit from pen.
47	3.9	11.0	1.4	No food for 4 days.

* Experiment of 4 hours. † Experiment of 3 hours.

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[6] Locke: *Journ. of Physiol.*, xxxvi, p. 205, 1907–08.

[7] Wilenko: *Arch. f. exp. Path. u. Pharm.*, lxxi, p. 261, 1913.

TABLE 2.

The Disappearance of Sugar from a Dextrose Solution Perfused through the Hydrazinized Rabbit's Heart.

NUMBER OF EXPERIMENT	BLOOD SUGAR CONTENT IN PERCENTAGES											WEIGHT OF HEART	TOTAL SUGAR DISAPPEARANCE	SUGAR DISAPPEARANCE PER GRAM HEART PER HOUR	REMARKS
	NORMAL	Hours after Hydrazine Administration													
		10	15	20	25	30	35	40	45	50	55	*grams*	*mgm.*	*mgm.*	
13	0.11		0.08									6.6	21.0	1.6	Liver not pale.
15	0.12			0.09								7.5	12.0	0.8	Liver very pale.
5	0.18			0.14			0.12					6.3	70.8*	2.8	Liver somewhat pale.
4	0.13			0.14			0.06					7.0	88.0†	3.1	Liver somewhat pale.
12	0.13			0.14			0.05					5.0	18.0	1.8	Liver very pale.
8	0.12			0.09				0.06				6.0	15.0	1.2	Liver not pale.
6	0.09			0.11				0.08				5.7	26.0	2.3	Liver not pale.
9	0.14								0.09			6.0	15.0	1.2	Liver very pale.
18	0.11								0.04		0.12	5.3	27.0	2.5	Liver slightly pale.
7	0.09					0.12					0.04	5.5	17.0	1.5	Liver slightly pale.
21	0.12	0.12										5.3	11.0	1.0	Liver normal.
23	0.16		0.13									4.0	16.0	2.0	Liver slate colored.
16	0.12			0.09	0.13							8.8	19.0	1.1	Liver normal.
10	0.14					0.10				0.10		5.0	27.0	2.7	Liver very pale.
20	0.14									0.10		4.6	21.0	2.2	Liver normal.
17	0.13									0.11		3.8	18.0	2.3	Liver normal.

* Experiment of 4 hours.

† Experiment of 3 hours.

Referring to the data in Table 2, the results of experiments with hydrazinized rabbits, it is at once apparent that no conclusive statement may be made since with these animals the figures obtained show an utter lack of uniformity.[8] At first it was thought that there existed a relation between the blood sugar content and the degree of sugar disappearance from the perfusion fluid, that is, that at a definite point in the decline of blood sugar content sugar transformation might be much more rapid than at any previous or subsequent period. The results as shown in Table 2 lend no support to such an hypothesis.

On the other hand, the data from experiments 10, 20 and 17, where in spite of hydrazine administration blood sugar content remained unchanged, furnish a possible clue to the significance of the distinctly augmented sugar disappearance from the perfusing fluid. In the experiments enumerated above the figures cited are much larger than those for the controls given in Table 1, and one may ask whether these larger figures obtained may not stand in direct relation to the nutritive condition of the animal, that is, with respect to its glycogen content or carbohydrate. To test this hypothesis experiments 33, 31, 34, 35, 32, 38, 36, and 39, Table 1, were carried through, and it is at once evident that simple starvation, without the intervention of hydrazine intoxication, is sufficient to account for the high figures now and then obtained in hydrazinized animals. If the lack of glycogen, one of the most obvious changes accompanying starvation, is alone responsible for the augmented sugar disappearance figures obtained one is at a loss to understand why the animals showing typical hydrazine symptoms, *i. e.*, low blood sugar, should exhibit figures for sugar disappearance characteristic of animals deviating little from the normal. Again, if the amount of glycogen is the sole determining factor it is difficult to understand why a period of three or four days' starvation, a period hardly sufficient to deplete entirely the glycogen store, should lead to results in sugar disappearance as great or greater than those obtained after a period of eight days' starvation.

Pursuing the idea of glycogen store in the tissues as a significant factor, experiments with well-fed animals were made, see Table 1, experiments 24, 26, 40, 41, 42, 43, and 37. It is evident from these results that the hearts of well-fed rabbits in general yield results significantly lower than those obtained with starved animals. It is also apparent that at times a rabbit in good nutritive condition will furnish a high figure, see experiment 42, Table 1.

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[8] Cf. results obtained by Patterson and Starling with the dog's heart, *Journ. of Physiol.*, xlvii, p. 137, 1913.

Another possible explanation for the behavior discussed above is the influence of the degree of contractility of the heart muscle. To eliminate so far as possible contractility of the heart muscle the hearts were perfused with calcium-free Locke's solution, see experiments 45, 48, 46, and 47. The figures obtained are indeed conflicting and afford little or no evidence in support of the idea that glycogen is the determining factor for increased sugar disappearance from the perfusion fluid. On the other hand, the data indicate definitely that the sugar disappearance is little different in the strongly beating heart than in the non-beating organ relaxed to its maximum, thus corroborating the conclusion of Locke.

CONCLUSIONS.

Hydrazine subcutaneously administered to rabbits causes no greater quantity of sugar to disappear from a solution perfused through the heart than that obtained with hearts of non-hydrazinized animals in a comparable nutritive condition.

With non-hydrazinized rabbits the nutritive condition, and hence possibly the quantity of glycogen present in the tissues, as indicated by starvation experiments, is apparently a factor in determining the sugar disappearance mentioned.

In confirmation of the work of previous investigators it is shown that sugar disappearance from the perfusion fluid with the beating heart is little different from that obtained when the heart is not beating and is relaxed to a maximum.

These experiments therefore fail to answer the question as to the cause of diminution of blood sugar content after hydrazine administration.

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THE CHEMISTRY OF GLUCONEOGENESIS.

VII. CONCERNING THE FATE OF PYRUVIC ACID IN METABOLISM.*[1]

BY A. I. RINGER.

(From the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia.)

The fate of pyruvic acid in the animal body has been the subject of considerable discussion in the past two years. P. Mayer[2] administered 7 to 8 grams of pyruvic acid to normal rabbits and found that the animals, when in a good state of nutrition, developed hyperglucaemia and glucosuria, while those that had been starved, and were therefore poor in glycogen content, developed hyperglucaemia only and no glucosuria. In those experiments he found that the urine often contained albumin after pyruvic acid administration, and further found that the administration of 10 to 15 grams brought about fatal intoxication. After pyruvic acid administration, he also found lactic acid in the urine.

In a second communication,[3] which appeared after our work was far advanced, Mayer reported the influence of pyruvic acid on gluconeogenesis of phlorhizinized dogs and rabbits. In none of his experiments was there an increase in the glucose elimination after pyruvic acid administration, while in two experiments on dogs (out of four) there was a very remarkable reduction in the glucose and nitrogen elimination. The kidneys of the dog in experiment 10 were examined microscopically and the following pathological conditions were found: "kalkhaltige Cylinder in den geraden Kanälen der Papille, Trübung und geronnene Massen in den Tubuli contorti, Hämoglobinniederschläge." He then drew the conclusion that py-

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* Received for publication, January 27, 1914.

[1] Aided by a grant from The Rockefeller Institute for Medical Research.

[2] P. Mayer: *Biochem. Zeitschr.*, xl, p. 441, 1912.

[3] P. Mayer: *ibid.*, xlix, p. 486, 1913.

ruvic acid is a toxic substance, which causes a depression in the nitrogen and glucose elimination by decreasing the permeability of the kidneys. He offered no explanation for the failure of pyruvic acid to bring about a decrease in the permeability of the kidneys in experiments 8 and 9, in which similar amounts of pyruvic acid were given, without any appreciable change in the glucose and nitrogen eliminations. In fact, there was a slight increase in both nitrogen and glucose output in experiment 9.

In a series of experiments reported by us[4] it was found that pyruvic acid, when given to phlorhizinized dogs *per os* or subcutaneously was not a toxic substance, and that it was glucogenetic. It is true that the glucogenetic properties of pyruvic acid were not found to be very constant, but in no case did we get the drop in nitrogen and sugar output as was observed by Mayer. Simultaneously with our communication Dakin and Janney[5] reported the results of their experiments from which they came to conclusions very similar to ours. They also found that pyruvic acid was glucogenetic, and in no case did they get a drop in the nitrogen and sugar output similar to that obtained by Mayer. Results similar to Dakin and Janney's and to ours have since been reported by Cremer.[6]

In three different laboratories experiments with pyruvic acid showed that it was glucogenetic and non-toxic. Mayer was the only one who obtained two negative results and two results which show very plainly that his pyruvic acid contained something that was toxic and had a peculiar effect upon the kidneys, an effect which resembles in its microscopical lesion as well as in its functional disturbance, the results that Underhill[7] and Pearce and Ringer[8] obtained after tartaric acid administration to phlorhizinized and normal dogs.

In his third communication on this subject[9] Mayer denies the presence of tartaric acid in his pyruvic acid, and suggests that the difference in our results may be due to polymerization of our pyruvic acid during the process of neutralization.

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[4] Ringer: this *Journal*, xv, p. 145, 1913.

[5] Dakin and Janney: *ibid.*, xv, p. 177, 1913.

[6] Cremer: *Berl. klin. Wochenschr.*, 1913, No. 31.

[7] Underhill: this *Journal*, xii, p. 115, 1912.

[8] Pearce and Ringer: *Journ. of Med. Research*, xxix, p. 57, 1913.

[9] P. Mayer: *Biochem. Zeitschr.*, lv, p. 1, 1913.

This explanation is not valid for two reasons: First, Dakin and Janney[10] have shown that polymerized pyruvic acid is non-glucogenetic. Second, we have, as Mayer has, observed great care in the process of neutralization. To eliminate all doubt, however, we performed one experiment in which pyruvic acid was administered subcutaneously unneutralized. As is seen from the record of the experiment, it possesses distinct glucogenetic properties, and has no toxic effect on the kidneys.

EXPERIMENT XXXIII.

Twelve-Hour Periods.

DATE Oct. 1913	PERIOD	WEIGHT	NITROGEN	GLUCOSE	D:N	EXTRA GLUCOSE	ACETONE AND ACETO-ACETIC ACID	β-HYDROXY-BUTYRIC ACID	REMARKS
15	XI	11.9	6.40	24.00	3.75		0.262	1.27	
15	XII		6.62	27.56	4.16	4.7	0.185	0.72	8.8 gms. of pyruvic acid dissolved in 3 cc. of olive oil given subcutaneously.
16	XIII	11.5	6.18	25.14	4.07		0.216	0.76	

We therefore still feel convinced that Mayer's results cannot be attributed to pyruvic acid, but to some extraneous influence.

THE FATE OF PYRUVIC ACID IN THE ANIMAL BODY.

In his first communication[11] Mayer showed that after the administration of pyruvic acid, *dl-* and *d*-lactic acid appeared in the urine. Embden and Oppenheimer[12] corroborated these findings. They perfused the extirpated surviving liver of dogs with blood to which pyruvic acid as ammonium or sodium salt had been added and found an increase in the lactic acid content of the perfused blood.

In another communication[13] Embden and Oppenheimer report their experiments on the influence of pyruvic acid on the formation of aceto-acetic acid in the perfused surviving liver of dogs. Twelve experiments were performed. Five gave no increase in aceto-acetic acid, and seven gave a very marked increase. They concluded that *pyruvic acid possesses the power of yielding aceto-acetic acid, because of the intermediary formation of acetaldehyde, which undergoes aldol condensation.*

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[10] Dakin and Janney: *loc. cit.*

[11] P. Mayer: *loc. cit.*

[12] Embden and Oppenheimer: *Biochem. Zeitschr.*, lv, p. 337, 1913.

[13] Embden and Oppenheimer: *ibid.*, xlv, p. 186, 1912.

From all this, we see that pyruvic acid can give rise to lactic acid on the one hand and to acetaldehyde on the other. In this connection it is important to remember that from Embden's experiments it is evident that *acetaldehyde is not always formed from pyruvic acid.*

In our experiments we found that *in some instances pyruvic acid yielded large quantities of glucose and in others it gave almost negative results.* On examining the relationship between the glucose formation and antiketogenesis in our experiments, a remarkable fact is evident: in case of high sugar formation from pyruvic acid, there is a marked depression in the acidosis (experiments XXII, XXIII and XXV); conversely, when there is little sugar formation, there is practically no change in the acidosis. Ringer and Frankel[14] have recently shown that when acetaldehyde is administered subcutaneously to phlorhizinized dogs, it possesses the power of causing an increase in the glucose elimination and a decrease in the acidosis.

On correlating all these facts it becomes evident that *pyruvic acid possesses its glucogenetic properties because acetaldehyde and lactic acid are formed in its intermediary metabolism.*

These two substances cannot possibly arise as a result of the same chemical process, and pyruvic acid must therefore be capable of following several paths of metabolism, as stated in our previous communication. The variable is not the pyruvic acid, but probably factors of equilibrium in the animal organism[15] and we believe there must exist a third possibility for the breakdown of pyruvic acid which results in no sugar formation. This would account for the very low sugar formation in experiments XXIV and XXVI of our series.[16]

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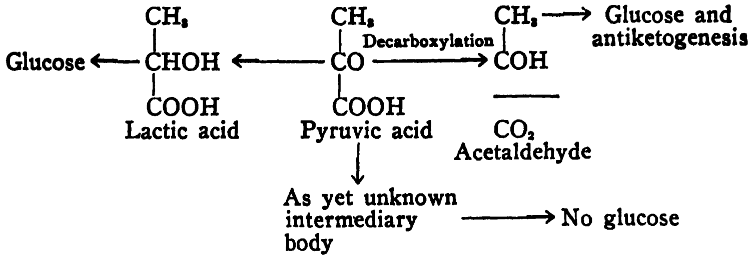
[14] Ringer and Frankel: this *Journal,* xvi, p. 563, 1914.

[15] Greer, Witzemann and Woodyatt: *ibid.,* xvi, p. 455, 1914.

[16] Ringer: *ibid.,* xv, p. 152, 1913.

We may therefore formulate *the fate of pyruvic acid in the animal organism* by the following scheme.



Another contribution recently appeared, also dealing with the question of sugar formation from pyruvic acid.[17] Its author, Barrenscheen, perfused the extirpated liver of a phlorhizinized dog with blood to which pyruvic acid as sodium salt had been added. He found no increase in the glucose concentration of the blood after perfusion, and he therefore concluded that pyruvic acid is not a glucogenetic substance.

From what was said above it becomes evident that the method of experimentation employed by Barrenscheen, is not at all adapted for settling this question. Since it was shown that the glucogenetic properties of pyruvic acid may be very largely due to the intermediary formation of acetaldehyde, and as it was also shown that in liver perfusions acetaldehyde undergoes aldol condensation with the formation of acetone bodies, whereas in the organism as a whole it causes the formation of extra glucose, the failure of the above author to find any increase in glucose in his experiment does not in any way lend support to Mayer's conclusions.[18]

Perfusion experiments with pyruvic acid, through the liver, may, however, become instructive if simultaneous analysis be made of the lactic acid, aceto-acetic acid and glucose concentration of the blood before and after the perfusion. This will show whether lactic acid is formed in those experiments where acetaldehyde fails to be formed, or whether the two substances are formed simultaneously.

[17] Barrenscheen: *Biochem. Zeitschr.*, lviii, p. 299, 1913.

[18] The same argument is applicable to the work of Parnas and Baer: *ibid.*, xli, p. 386, 1912.

[Reprinted from the PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE, April 15, 1914, Vol. xi, No. 4, pp. 124–125.]

ON THE HEXOSAMINE OF CHONDROITIN SULPHURIC ACID.

BY P. A. LEVENE AND F. B. LA FORGE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In a previous communication[1] the writers reached the conclusion that the nitrogenous component of chondroitin sulphuric acid was glucosamine. The conclusion was based on the analytical data of the hydrochloride of the amino sugar, and on the magnitude of its optical rotation.

However, recently it was discovered that the optical activity of the amino sugar differed considerably from that of glucosamine, if measured under very definite conditions. The conditions required are the following: low temperature of the solution, comparatively high concentration of the sugar solution, and measuring the initial rotation immediately after the solution of the sugar is accomplished. Under such conditions it was found that the specific rotation of the amino sugar of the chondroitin sulphuric acid was about 25 per cent. higher than that of glucosamine. Both substances dis-

Substance.	Crystal-form.	Rotation.		Solubility.	Melting Point.
		Initial.	Final.		
		$[\alpha]_{D}0^{\circ} =$			
Glucosamine hydrochloride.........	Short thick prisms.	+101.6	+73.65	Difficultly sol. in 80 per cent. EtOH; insol. in abs. EtOH.	Decomposes slowly above 200°.
Osazone..........	Needles.			Difficultly sol. in abs. EtOH.	Melts with decomposition 206°.
Hexosamine hydrochloride.........	Long prismatic needles.	+129.5	+93.82	Easily sol. in 80 per cent. EtOH; difficultly sol. in abs. EtOH.	Melts with decomposition 180°.
Osazone..........	Long needles.			Easily sol. in EtOH; sol. in hot H_2O.	175–180°.

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[1] *Jour. Biol. Chem.*, XV, p. 155, 1913.

played mutirotation and reached equilibrium simultaneously. Also in its melting point and in its solubility the amino sugar differed from glucosamine. Finally the osazones of the two substances differed in their melting points and in their solubility in alcohol and in water.

On the basis of these data it seems justified to conclude that the hexosamine is not identical with glucosamine, but is isomeric to it.

Work on the configuration of the sugar is in progress.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, May, 1914, Vol. xvii, No. 4, pp. 443–449.]

THE ACTION OF LEUCOCYTES AND KIDNEY TISSUE ON PYRUVIC ACID.*

BY P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

The knowledge of the mechanism of "glycolysis" in animal tissues remains very meager. Definitely established is the formation of lactic acid in one of the intermediate phases of the process. It is probable that the latter phase is preceded by the appearance of pyruvic aldehyde. The phases which follow the formation of lactic acid and which lead to the production of carbon dioxide and of water are not known.

The mechanism of sugar metamorphosis in plants, and particularly in yeast, has been studied more successfully. Recently Neuberg[1] and his collaborators have brought forward a number of observations which indicate that in the process of alcoholic fermentation pyruvic acid is formed as an intermediate stage. This substance then dissociates into carbon dioxide and acetaldehyde, according to the following reaction.

$$CH_3CO.COOH = CO_2 + CH_3COH$$

It seemed possible that also in the animal organism the conversion of hexose into carbon dioxide and water is preceded by the formation of pyruvic acid. Tschernorutzky,[2] working in Neuberg's laboratory, offered evidence in support of this hypothesis. This worker has claimed that animal tissues possess the power of cleaving pyruvic acid in a manner leading to the formation of carbon dioxide. However, Tschernorutzky failed to furnish satisfactory evidence

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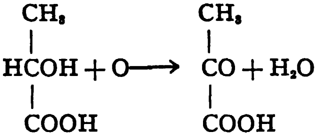
* Received for publication, March 13, 1914.

[1] *Biochem. Zeitschr.*, xxxvii, p. 170, 1911; *ibid.*, liii, p. 406, 1913.

[2] *Ibid.*, xliii, p. 486, 1912.

that bacteria did not play the part ascribed by him to the tissues. Animal experiments with feeding pyruvic acid were performed by Paul Mayer, Dakin, Ringer and Embden[3] and on the basis of their work one feels justified in assuming that pyruvic acid may be formed in course of the many transformations which dextrose suffers in the organism.

In our previous experiments with aseptic kidney tissue, or with leucocytes we were unable to bring about an oxidation of lactic acid. Since *a priori* it is possible that two specific enzymes are required for the final oxidation of lactic acid into carbon dioxide and water: namely, one transforming lactic acid to pyruvic acid according to the following reaction:



$$\begin{array}{c} CH_3 \\ | \\ HCOH \\ | \\ COOH \end{array} + O \longrightarrow \begin{array}{c} CH_3 \\ | \\ CO \\ | \\ COOH \end{array} + H_2O$$

and the second leading to formation of carbon dioxide, it seemed possible that the kidney tissue and the leucocytes did not possess the first enzyme and yet had the second. For this reason we concluded to repeat the experiments of Tschernorutzky under absolutely aseptic conditions. The experiments were not performed with liver tissue for the reason that it is practically impossible to obtain liver tissue free from bacteria.

Very recently we noted that Ida Smedley MacLean[4] had also been engaged in work on the same problem but has temporarily abandoned the investigation for the lack of an adequate analytical method.

The conclusions regarding the fate of pyruvic acid were based first on the production or non-production of carbon dioxide in the course of the reaction, and second on the behavior of the reaction mixtures towards potassium permanganate.

It was reasoned that if the decomposition of pyruvic acid in animal tissues proceeds in the same manner as in the yeast, the reaction remaining at the acetaldehyde phase, then the oxygen requirement for final transformation of the products into carbon dioxide and water remains the same as for the conversion of pyruvic acid. This is made evident from the following:

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[3] Mayer: *Biochem. Zeitschr.*, xlix, p. 486, 1913; Dakin and Janney: this *Journal*, xv, p. 177, 1913; Ringer: *ibid.*, xv, p. 145, 1913; Embden and Oppenheimer: *Biochem. Zeitschr.*, lv, p. 335, 1913.

[4] *Biochem. Journ.*, vii, p. 611, 1913.

$$CH_3{\cdot}CO{\cdot}COOH + 5\,O = 3CO_2 + 2H_2O$$
$$CH_3{\cdot}COH + 5\,O = 2CO_2 + 2H_2O$$

For either one of the two reactions five atoms of oxygen are required.

If, however, pyruvic acid, or the products of dissociation suffered oxidation, or if the acetaldehyde underwent reduction, then the oxygen requirement for final conversion of the products into carbonic acid and water would differ from that for pyruvic acid.

It was found that the yeast enzyme failed to act on pyruvic acid at 37°C., but at 42° caused intense evolution of carbon dioxide as stated by Neuberg and coworkers. Hence our experiments were conducted at temperatures varying between 37° and 42°C.

However, in all the experiments, provided they remained perfectly aseptic, there was never observed either the formation of carbon dioxide or a change of the oxygen requirement for transformation of the reaction product into carbon dioxide and water. Hence there is no reason to assume that under conditions of perfect asepsis leucocytes or kidney tissue are capable of decomposing pyruvic acid.

It may be mentioned here that the presence of pyruvic acid does not in any way affect the accurate estimation of carbon dioxide by means of distillation under diminished pressure. Also by means of a barium hydroxide solution it is possible to remove accurately all the carbon dioxide in the presence of pyruvic acid if the operation is performed at low temperature. However, it was not found possible to apply the method in the present work for the reason that in the presence of the autolytic products of the tissues the barium carbonate precipitate was quite flocculent and mechanically carried with it small quantities of pyruvic acid.

The bacteriological part of this work was done by Dr. Martha Wollstein, to whom we wish to acknowledge our indebtedness.

It may be also mentioned here that the evolution of carbonic acid gas was only observed in some and not in all experiments contaminated with bacterial growth.

EXPERIMENTAL.

Tissues.—Leucocytes were obtained from dogs by the injection of turpentine into the pleural cavity by the method described in a previous communication. Kidneys from dogs and rabbits were used. They were removed aseptically from exsanguinated animals.

Solutions.—Pyruvic acid was prepared according to the method of Simon,[5] and refractionated several times. It boiled constant at 68°, under 11 mm. pressure. In order to obtain all solutions sterile without decomposing the pyruvic acid it was necessary to sterilize the pyruvic acid, sodium hydrate and Henderson phosphate solutions separately and then mix them immediately prior to adding the leucocytes or the minced kidneys. The substances were always used in the following proportions: Pyruvic acid, 1.0 gram, Henderson phosphate solution, 100 cc. and 9 cc. of a 5 per cent sodium hydroxide solution. The sodium hydroxide was prepared very carefully and was freed from carbonates.[6] Inasmuch as the controls had to be brought to a boil to destroy any enzymes and as this would also decompose the pyruvic acid, a somewhat different procedure was necessary. The kidney pulp was added to the phosphate solution, this brought to a boil, and when cold the pyruvic acid was added which had previously been neutralized with sodium hydroxide. The tissue mixture was allowed to stand at 37°, 40° and 42° for seven days.

Bacteriological Controls.—Smears and cultures were made of all tissue mixtures prior to analysis and only those free from all contamination were considered.

METHODS OF ANALYSIS.

Oxidation with Permanganate.—The total oxidation as described by Greifenhagen, König and Scholl[7] and also used by us in a previous investigation[8] was used and found to give theoretical values with pyruvic acid. The tissue mixtures, after straining through cheese cloth, were freed from protein with metaphosphoric acid and the filtrate made up to 500 cc. Of this 20 cc. were used for each oxidation.

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[5] *Bull. soc. chim.*, 1895, p. 335.

[6] Cowles, H. W. Jr.: *Journ. Amer. Chem. Soc.*, xxx, p. 1192, 1908.

[7] *Biochem. Zeitschr.*, xxxv, p. 176, 1911.

Carbon Dioxide Determination.—It was found that in the cold pyruvic acid is not precipitated nor decomposed by dilute barium hydroxide. The tissue mixtures were strained through cheese cloth and the filtrate thoroughly cooled by freezing mixtures of ice and alcohol. Barium hydroxide solution (10 per cent) previously filtered was likewise cooled and then added to the above until no further precipitate was formed. This was then rapidly filtered in the cold on a Buchner funnel. The funnel was covered to protect the solution from carbon dioxide of the atmosphere. The precipitate was thoroughly washed with ice-cold water and then together with the filter paper put into a flask for the determination of carbon dioxide as described in a previous communication.[9] As already mentioned, this method gave too high carbon dioxide values as pyruvic acid was carried down mechanically with the carbonate in the presence of autolytic products.

Carbon Dioxide Determination under Diminished Pressure.—The protein solution containing pyruvic acid, with a few drops of alizarin as indicator, was placed in a double necked distilling flask arranged for vacuum distillation. The distilling flask was fitted with a capillary and also a dropping funnel for introducing phosphoric acid without opening the flask. The protruding end of the capillary was connected by rubber tubing to a series of wash bottles containing strong solutions of sodium hydroxide and finally barium hydroxide as an indicator that all air entering the capillary was freed of carbon dioxide. The further arrangement of the flasks was very similar to that used for the determination of ammonia[10] under diminished pressure, only, in place of the third distilling flask being attached directly to the source of vacuum, three wash bottles, two of the Drexel and one of the Wetzlar pattern, were interposed.

[8] This *Journal,* xii, p. 268, 1912.

[9] This *Journal,* ix, p. 101, 1911.

[10] Van Slyke: *ibid.,* x, p. 20, 1911.

The second distilling flask was kept empty; the third flask and succeeding wash bottles contained measured amounts of $\frac{N}{5}$ barium hydroxide. After all flasks and bottles were joined, the system was evacuated and only then was the phosphoric acid admitted by the dropping funnel to acidify the solution under examination. It was found on known carbonate solutions that the carbon dioxide was entirely removed from the flask in one hour with the corresponding formation of barium carbonate in the first two and rarely in the last two receiving flasks or bottles, and this occurred only when large amounts of carbonate were analyzed. To avoid any error the distillation always lasted two hours. A vacuum of 15 to 20 mm. was always maintained and the flask containing the pyruvic acid never warmed beyond 25°. Care must be taken in discontinuing the vacuum that the barium hydrate containing the carbonate is not sucked back, which is best accomplished by closing the stop cock from the vacuum supply and opening the separatory funnel simultaneously. The amount of carbon dioxide admitted with the air in this way is entirely negligible. The barium hydrate is then filtered through a Gooch crucible from the carbonate and the filtrate titrated with $\frac{N}{10}$ hydrochloric acid, using the proper precautions to exclude carbon dioxide of the air.

ANALYTICAL RESULTS.

Inasmuch as the results all indicate that pyruvic acid is not decomposed by aseptic animal tissues, as there was no change in the oxidation value or an increase of carbon dioxide over the controls, only a few of the numerous analyses are here recorded.

Oxidation with Potassium Permanganate.

Pyruvic Acid.—1 cc. $\frac{N}{5}$ $KMnO_4 = 1.801$ mgm. pyruvic acid. 20 cc. of a 0.2 per cent = 0.040 mgm. pyruvic acid solution, were oxidized with 40 c.c. $\frac{N}{5}$ $KMnO_4$ and 60 cc. 10 per cent KOH. After acidifying with 50 cc. 25 per cent H_2SO_4, 18 cc. $\frac{N}{5}$ oxalic acid were required for titration, therefore 22 cc. $\frac{N}{5}$ $KMnO_4$ were utilized for oxidation = 0.0396 mgm. pyruvic acid.

Pyruvic Acid and Leucocytes.—(1) Kept at 37° for one week. (2) Control with boiled leucocytes kept at 37° for one week. Both freed of protein with metaphosphoric acid and filtrate made up to 500 cc.

	CC. USED	$\frac{N}{5}$ KMnO$_4$	$\frac{N}{5}$ OXALIC ACID	$\frac{N}{5}$ KMnO$_4$ CC. USED	PYRUVIC ACID
		cc.			*mgm.*
1	20	40	17.20	22.80	41.06
2	20	40	17.30	22.70	40.88

Pyruvic Acid and Rabbit Kidneys.—(1) Kept at 37° for one week. (2) Control, boiled and kept at 37° for one week. Both freed of protein with metaphosphoric acid and filtrate made up to 500 cc.

	CC. USED	$\frac{N}{5}$ KMnO$_4$	$\frac{N}{5}$ OXALIC ACID	$\frac{N}{5}$ KMnO$_4$ CC. USED	PYRUVIC ACID
		cc.			*mgm.*
1	20	40	17.30	22.70	40.80
2	20	40	17.10	22.90	41.40

Pyruvic Acid and Rabbit Kidneys.—(1) Kept at 45° for one week. (2) Control, boiled and kept at 45° for one week. Both freed of protein with metaphosphoric acid and filtrate made up to 500 cc.

	CC. USED	$\frac{N}{5}$ KMnO$_4$	$\frac{N}{5}$ OXALIC ACID	$\frac{N}{5}$ KMnO$_4$ CC. USED	PYRUVIC ACID
		cc.			*mgm.*
1	20	40	14.60	25.40	45.74
2	20	40	14.50	25.50	45.92

Determination of Carbon Dioxide under Diminished Pressure.—The carbon dioxide was always determined on the total quantity of the mixture of tissue and pyruvic acid.

1. (a) Solution of sodium carbonate. 50 cc. were used.
   (b) CO_2 determined by steam distillation process.
2. Pyruvic acid + NaOH + Henderson phosphate solution.
3. (a) Pyruvic acid, 1 per cent, and rabbit kidney kept at 42° for one week.
   (b) Control, boiled and kept at 42° for one week.
   (c) Solution 3 (a) to which 25 cc. of the sodium carbonate solution mentioned above in (1) were added after the first CO_2 estimation was terminated.
4. (a) Pyruvic acid, 0.5 per cent and rabbit kidneys kept at 37° for one week.
   (b) Control, boiled and kept at 37° for one week.
5. (a) and (b) Same as 4 (a) and (b) except 1 per cent pyruvic acid.

	$\frac{N}{10}$ Ba(OH) CC. USED	$\frac{N}{10}$ HCl CC. USED	DIFFERENCE	MGM. CO_2
1 (a)	78.00	61.50	16.50	36.30
(b)	60.00	43.30	16.70	36.70
2	20.00	20.00		
3 (a)	24.00	22.60	1.40	3.08
(b)	24.00	22.80	1.20	2.68
(c)	60.00	51.80	8.20	18.04
4 (a)	22.80	20.00	2.80	6.16
(b)	22.80	20.00	2.80	6.16
5 (a)	22.80	20.40	2.40	5.28
(b)	22.80	20.40	2.40	5.28

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THE CHEMISTRY OF GLUCONEOGENESIS.

VIII. The Velocity of Formation and Elimination of Glucose by Diabetic Animals.*[1]

By A. I. RINGER and E. M. FRANKEL.

(From the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia.)

In the study of the glucogenetic properties of the fatty acids and their derivatives, it was frequently observed that after subcutaneous or oral administration of the substance, the complete yield of "extra" glucose was not obtained within the first period of twelve hours. This retardation in the appearance in the urine of the "extra" glucose may be due to one of the following causes: 1. Slow absorption of the substance fed. 2. Slow rate of the glucose formation. 3. Slow elimination of the glucose by the kidneys. It was with a view to determining which of these factors was the underlying cause that these experiments were undertaken.

To eliminate the factor of absorption the substances were administered intravenously (saphenous vein) and the nitrogen and glucose in the urine were determined in short periods of two or three hours, as indicated in the tables. The "extra" glucose eliminated was computed by assuming the mean D:N ratio of the fore and after periods of twelve hours.

The animals were phlorhizinized by the daily injection of 1 gram of phlorhizin ground up in olive oil. The urine was collected by catheter and the bladder washed thoroughly with distilled water at the end of each period. This is very important when the periods are short.

In experiment XXXIV, 7.4 grams ($\frac{M}{10}$) of propionic acid, neutralized with sodium hydroxide, were dissolved in 150 cc. of isotonic

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* Received for publication, April 15, 1914.

[1] Aided by a grant from The Rockefeller Institute for Medical Research.

salt solution and slowly injected intravenously at the beginning of period B. No anesthesia was found necessary for these operations.

The D:N ratio in the fore period of twelve hours was 2.62 and in the after period was 3.04; we therefore assume the mean of these ratios, 2.83, as the one which would have persisted if no propionic acid had been administered. This assumption is borne out by the fact that the D:N ratio in the one-hour fore period (A) was 2.76. By multiplying the nitrogen of each period by 2.83 we found the amount of glucose that originated from the catabolized protein. And by subtracting this figure from the total glucose, the amount of "extra" glucose was obtained.

During period B, the first two hours after the propionic acid injection, 2.34 grams of "extra" glucose were eliminated. In period D there was a considerable drop in the amount of "extra" glucose elimination, amounting to only 0.96 gram, and in periods E and F, 0.83 and 0.82 gram were eliminated respectively. The total amount of "extra" glucose eliminated in the eleven hours was 7.83 grams. This is considerably less than was found previously[2] after subcutaneous or oral administration. The difference may be explained by the rapid entrance of propionic acid into the blood and the excretion of some by the kidneys before it is converted into glucose.

In experiment XXXV a similar amount of propionic acid was similarly injected intravenously at the end of the first hour of period A. The D:N ratios of the fore and after periods of twelve hours were 2.32 and 2.91. In the calculation of the "extra" glucose we assumed the mean of the two, which is 2.61. The correctness of this assumption is again borne out by the fact that the D:N ratios in periods E and F were 2.53 and 2.51 respectively.

During the second hour of period A, *i.e.*, the first hour after the propionic acid administration, there was an elimination of 0.25 gram of "extra" glucose. In period B 1.94 grams and in period C 2.66 grams were eliminated. In period D there was a marked decline; only 0.84 gram was eliminated. The total amount of "extra" glucose eliminated was 5.69 grams.

In experiment XXXVI, 9 grams ($\frac{M}{20}$) of glucose were dissolved in 150 cc. of isotonic salt solution and injected intravenously at the end of the first hour of period A. The D:N ratios of the fore and after periods were 3.23 and 3.77 respectively. The mean of 3.55 was used in computing the "extra" glucose. During the second hour of period A, *i.e.*, the first hour after the glucose administration, 3.91 grams of "extra" glucose were eliminated. In period B 2.47 grams of "extra" glucose were eliminated and in period C there was 0.39 gram of "extra" glucose. The total amount of "extra" glucose eliminated was 6.77 grams.

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[2] Ringer: this *Journal*, xii, p. 511, 1912.

In experiment XXXVII, 9 grams of glucose ($\frac{M}{20}$) dissolved in about 50 cc. of water were given *per os* at the beginning of period B. During the first two hours (period B) 5.32 grams of "extra" glucose were eliminated, and 2.34 grams were eliminated in period C. In periods D and E 0.68 and 0.37 grams of "extra" glucose were eliminated. The total elimination of "extra" glucose was 8.71 grams.

DISCUSSION OF RESULTS.

The curves showing the velocity of the "extra" glucose elimination are plotted for the purpose of bringing out more clearly the results of these experiments. They show with what explosive rapidity the glucose is excreted by the kidneys after it enters the blood stream. The absorption of the glucose from the gastro-intestinal tract, in experiment XXXVII, must be exceedingly rapid, for the curve during the first two hours scarcely differs from that of experiment XXXVI.

The curves of "extra" glucose elimination, after glucose administration, are very typical and differ materially from those which follow propionic acid administration. The former passes the 5-gram mark in less than two hours, whereas the corresponding point on the propionic acid curve is touched in four to five hours. Since factors of absorption do not play any rôle here, *the difference in the time relationship can be attributed only to the time required for the synthesis of glucose from propionic acid.*

These experiments justify the conclusions that:

1. There is no delay in glucose elimination by the kidneys, but it is excreted almost as fast as it enters the blood stream.

2. In the slow elimination of "extra" glucose, factors of absorption and velocity of sugar formation may play contributing rôles.

EXPERIMENT XXXIV.

Dog's weight 10.85 kgm.

PERIOD	NUMBER OF HOURS	NITROGEN	NITROGEN PER HOUR	GLUCOSE	GLUCOSE PER HOUR	D:N	"EXTRA" GLUCOSE	REMARKS
VI	12	3.46	0.288	9.07	0.756	2.62		7.4 grams of propionic acid as sodium salt, dissolved in 150 cc. of isotonic salt solution, injected intravenously at the beginning of period B.
VII A	1	0.36	0.360	0.993	0.993	2.76		
B	2	0.716	0.358	4.370	2.185	6.10	2.34	
C	2	0.860	0.430	5.31	2.650	6.18	2.88	
D	2	0.723	0.362	3.01	1.505	4.16	0.96	
E	2	0.567	0.284	2.43	1.215	4.29	0.83	
F	3	0.690	0.230	2.77	0.925	4.02	0.82	
Total for per. VII		3.916		18.883		4.81	7.83	
VIII	12	2.79	0.232	8.52	0.710	3.04		

EXPERIMENT XXXV.

Dog's weight 6.80 kgm.

PERIOD	NUMBER OF HOURS	TOTAL NITROGEN	NITROGEN PER HOUR	TOTAL GLUCOSE	GLUCOSE PER HOUR	D:N	"EXTRA" GLUCOSE	REMARKS
X	12	4.050	0.337	9.37	0.781	2.32		At end of first hour of period A 7.4 grams of propionic acid as sodium salt dissolved in 150 cc. of isotonic salt solution, ininjected intravenously.
XI A	2	0.684	0.342	2.03		2.98	0.25	
B	2	0.245	0.122	2.58	1.290	10.54	1.94	
C	2	0.668	0.334	4.40	2.200	6.57	2.66	
D	2	0.968	0.484	3.37	1.685	3.49	0.84	
E	2	0.802	0.401	2.03	1.015	2.53		
F	2	0.704	0.352	1.759	0.879	2.51		
Total for per. XI		4.071		16.169		3.97	5.69	
XII	12	3.01	0.251	8.75	0.729	2.91		

EXPERIMENT XXXVI.

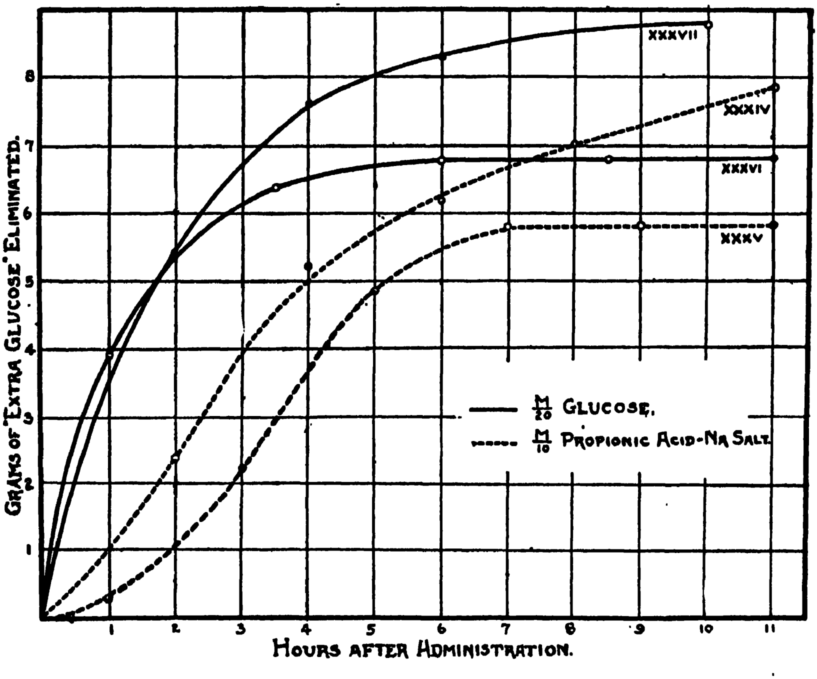
Dog's weight 10.49 kgm.

PERIOD	NUMBER OF HOURS	TOTAL NITROGEN	NITROGEN PER HOUR	TOTAL GLUCOSE	GLUCOSE PER HOUR	D:N	"EXTRA" GLUCOSE	REMARKS
X	12	3.04	0.253	9.83	0.819	3.23		At end of first hour of period A, 9 grams of glucose dissolved in 150 cc. of isotonic salt solution, injected intravenously.
XI A	2	0.608	0.304	6.04		9.94	3.91	
B	2.5	0.717	0.287	4.98	1.990	6.95	2.47	
C	2.5	0.558	0.223	2.34	0.936	4.20	0.39	
D	2.5	0.564	0.225	1.95	0.783	3.47		
E	2.5	0.575	0.230	2.07	0.828	3.60		
Total for per. XI		3.022		17.38		5.76	6.77	
XII	12	2.68	0.223	10.09	0.841	3.77		

EXPERIMENT XXXVII.

Dog's weight 9.48 kgm.

PERIOD	NUMBER OF HOURS	TOTAL NITROGEN	NITROGEN PER HOUR	TOTAL GLUCOSE	GLUCOSE PER HOUR	D:N	"EXTRA" GLUCOSE	REMARKS
I	12	5.05	0.420	16.28	1.36	3.22		
II	12	4.65	0.387	14.50	1.21	3.12		9 grams of glucose dissolved in about 50 cc. of water given *per os* at beginning of period B.
III A	2	0.614	0.322	2.280	1.140	3.72		
B	2	0.728	0.364	7.616	3.808	10.48	5.32	
C	2	0.583	0.291	4.176	2.088	7.17	2.34	
D	2	0.668	0.334	2.789	1.394	4.19	0.68	
E	4	1.270	0.318	4.376	1.094	3.44	0.37	
Total for per. III		3.86		21.237		5.50	8.71	
IV	12	3.41	0.284	10.90	0.909	3.20		
V	12	3.70	0.308	11.66	0.972	3.15		


XXXVII
XXXIV
XXXVI
XXXV
M/20 Glucose.
M/10 Propionic Acid-Na Salt.
Grams of "Extra Glucose" Eliminated.
1
2
3
4
5
6
7
8
Hours after Administration.
1
2
3
4
5
6
7
8
9
10
11

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ON CHONDROITIN SULPHURIC ACID.

THIRD PAPER.*

BY P. A. LEVENE AND F. B. LA FORGE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

By the results of our previous work it was demonstrated that two carbohydrate derivatives occur in chondroitin sulphuric acid.[1] One was recognized as *d*-glucuronic acid and the other as a hexosamine. The hexosamine, in the form of its crystalline salt of hydrochloric acid, was obtained directly on concentration of the product of hydrolysis of chondroitin sulphuric acid. The specific rotation of this salt, measured in 1 per cent solution at room temperature and in pure yellow light, was found to be 99°. There was at the time only one hexosamine known, glucosamine, which is best identified as the hydrochloric acid salt. The initial specific rotation of this salt is given as + 100°. Hence, it was assumed that the hexosamine obtained from chondroitin sulphuric acid was identical with glucosamine.

Since then, the properties of the substance were scrutinized in greater detail, and the new information compels us to abandon the view of the identity of the new hexosamine with glucosamine. The substance is isomeric with glucosamine, and until the details of its structure will be disclosed it will be referred to as chondrosamine. Chondrosamine resembles glucosamine in its elementary composition and differs from it in the physical properties of its derivatives. A comparative study was made of the following derivatives: the hydrochloric acid salts, the phenylosazones, the dicarboxylic acids obtained on oxidation of the amino sugars by means of nitric acid, and of the monocarboxylic acids obtained on oxidation with bromine.

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* Received for publication, May 5, 1914.

[1] This *Journal*, xv, p. 69 and p. 155, 1913.

The hydrochlorides differ in their crystal form, in their solubility in water and in dilute alcohol, in their melting points, and their specific rotations. The salt of glucosamine is quite soluble in water, almost insoluble in 80 per cent alcohol; the salt of the new substance is quite soluble in 80 per cent alcohol. The salt of glucosamine showed no tendency to melt at a temperature considerably above 200°C., whereas the melting point of the hydrochloride of chondrosamine is sharp at 182°C. The optical rotation of the two substances is particularly worthy of note. That of the salt of chondrosamine has an initial specific rotation of 129°, is mutarotating and reaches the equilibrium at 93°; glucosamine hydrochloride also is mutarotating, with the initial rotation of 100° and the equilibrium point at 73°.

If the rotation of chondrosamine is measured at room temperature and in 1 per cent solution it may show the effect of mutarotation already at the time of the first reading. This occurred in our first experiments with the substance, and the occurrence misled us into the belief that chondrosamine possessed the specific rotation of glucosamine. In order to avoid this possible error the first reading should be taken immediately after solution of the substance is effected and precautions should be exercised to prevent the temperature of the solution from rising above 0°C.

The osazones differ in their melting points, and in their specific rotation. The phenylosazone of glucosamine is regarded as identical with glucosazone. A sample prepared by us had a melting point of 208°C., possessed the solubility of glucosazone and hence could be recrystallized out of 98 per cent alcohol. The initial rotation under the conditions indicated by Neuberg was —0.62° and the equilibrium —0.35°. The phenylosazone of chondrosamine is very soluble even in dilute alcohol and has to be recrystallized out of water. The melting point of the substance is depressed apparently by very slight impurities. The highest melting point was obtained only when measured on a very fresh sample of the substance, that had not been exposed to the light. The highest melting point obtained was 180° with decomposition at rapid heating.

It must be noted also that chondrosamine forms apparently the same osazone if it has been deaminized prior to the treatment with phenylhydrazine. According to Fischer and Tiemann,[2] glucosamine if deaminized yields only an insignificant quantity of osazone, and Tiemann assumed that this was due to the fact that deaminization was incomplete. The melting point of the osazone obtained from deamino-chondrosamine showed the identical melting point with the substance obtained directly from the amino sugar. However, this was attained only by repeated recrystallization. After one recrystallization the substance melts at about 160°C. and shows a decomposition point at 180°C.

The initial rotation of two samples of the osazone, one formed directly from the sugar and the other from the amino sugar. showed a discrepancy, but the equilibrium rotation was practically identical and both different in magnitude and in direction from that of glucosazone: namely, the initial rotation was + 0.60° for the first and + 0.45° for the second, the equilibrium rotations being + 0.20 and + 0.18° respectively.

The dicarboxylic acids were analyzed in form of their calcium salts. The most striking difference was found in their specific rotation. Isosaccharic acid prepared by us showed an initial rotation of + 0.20° and final rotation of + 0.06°. The acid obtained from the new sugar showed an initial rotation of — 0.45° and final of — 0.37°. The acid will be referred to as "chondrosic acid."

Monocarboxylic acid of the new sugar will be referred to as chondronic acid. It was obtained and analyzed in form of its brucine salt. The substance melted at 213° and had a specific rotation of 16.85°. No corresponding salt of chitonic acid has been described.

Discussion of Results.—The results here enumerated permit only of the conclusion that chondrosamine is isomeric with glucosamine. The fact that in the process of the osazone formation the amino group is eliminated from the molecule, renders it probable that the group is attached to the α-carbon (to the carbonyl group). The details of the structure of chondrosamine will be established by further investigation. Work in this direction is now in progress.

In conclusion we wish to refer again to an observation made in Hofmeister's laboratory. Kondo[3] prepared on treatment of the products of hydrolysis of chondroitin sulphuric acid an osazone having a melting point at 143°C. Discussing this observation Kondo referred to the similarity of the osazone with that of xylose. We inferred that the discussion indicated the author's inclination to regard xylose as a probable constituent of chondroitin sulphuric acid. Since then Professor Hofmeister informed us in private correspondence that both he and his co-workers were convinced that the similarity of their osazone with xylosazone was only superficial, and that we attached undue emphasis to their discussion. At present it seems to us probable that their osazone was that of chondrosamine, but in a state of imperfect purity.

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[2] *Ber. d. deutsch. chem. Gesellsch.*, xxvii, p. 140, 1894.

[3] *Biochem. Zeitschr.*, xxvi, p. 116, 1910.

EXPERIMENTAL.

Preparation of Hexosamine Hydrochloride.

Seventy-five grams of chondroitin sulphuric acid barium salt were hydrolyzed for about seven and a half hours with 400 cc. of 20 per cent hydrochloric acid, with the addition of 15 grams of stannous chloride. Barium sulphate began to separate at once and soon the solution began to take on a yellow color which passed rapidly through brown to black with the separation of dark particles due to decomposition of glucuronic acid. Upon completion of the reaction the solution was diluted with twice its volume of warm water and without filtering the tin removed with hydrogen sulphide. The sulphides of tin were separated by filtration with suction, leaving a clear, almost colorless filtrate which, without further treatment, was concentrated in vacuum to about 35 cc. This syrup-like residue was at once taken up in 75 to 80 cc.[4] of absolute alcohol, poured into a beaker and the hydrochloride of the amino hexose caused to crystallize by adding about 100 cc. of absolute ether slowly in portions of about 10 cc. with constant scratching of the sides of the vessel. The deposit of long white prismatic needles thus obtained was filtered with suction and washed with absolute alcohol and ether. The first yield usually amounts to about 16 grams while upon addition of 50 c.c. more of ether to the first filtrate about 4 grams more of equally pure product are obtained. The total yield corresponds to about 90 per cent of the theory. Upon recrystallization under the above conditions or on long keeping in a desiccator, the product tends to lose a small amount of hydrochloric acid, since an analysis of a product twice so treated gave the following figures:[5]

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[4] The presence of too much alcohol or water causes the product to separate oily at first.

0.1794 gram of substance required 14.6 cc. $AgNO_3$ solution (1 cc. = 0.00186 gram Cl).

0.1701 gram of substance gave 0.0977 gram H_2O and 0.2113 gram CO_2.

	Calculated for $C_6H_{13}O_5N.HCl$.	Found.
Cl	16.45	15.15
C	33.40	33.93
H	6.54	6.55

The optical determination was carried out at 0°. All apparatus was cooled to this temperature. 0.3000 gram of substance in 3 cc. H_2O, total weight of solution 3.2871 grams, specific gravity 1.0352, in 0.5 dcm. tube.

After five minutes $+ 6.10°: [\alpha]_D^0 = 129.50°$.

After twenty-four hours $+ 4.44°: [\alpha]_D^0 = 93.82°$.

The activity of pure glucosamine was determined for comparison under the same condition. 0.3000 gram of substance in 3 cc. H_2O, weight of solution 3.2876 grams, specific gravity 1.0327, in 0.5 dcm. tube.

After five minutes $+ 4.79°: [\alpha]_D^0 = 101.60°$.

After twenty-four hours $+ 3.50°: [\alpha]_D^0 = 73.65°$.

Phenylosazone from Deamino Chondrosamine.

Three grams of the hexosamine hydrochloride were deaminized with 3 grams of silver nitrite with the addition of a few drops of hydrochloric acid. After about three hours, at room temperature, the silver chloride was filtered off and an excess of silver in the filtrate removed by treatment with hydrogen sulphide. The solution was then boiled to remove the excess of the latter and heated for one and a half hours on the water bath with 6 grams of phenyl-hydrazine in 20 cc. of glacial acetic acid and 3 grams of sodium acetate. It was then diluted to 300 cc. with hot water and filtered. Upon cooling the osazone deposits in the filtrate in long yellow needles which were after a time filtered off and recrystallized from 300 cc. of water. The yield of the first product was over 1 gram. The substance melts at about 160° and decomposes at about 180°. Again recrystallized the melting point rose to 175° with decomposition at about 185°. Upon keeping, especially if not quite pure, the product deteriorates rapidly with superficial decoloration.

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[5] Normal figures are obtained if the substance is crystallized from alcohol and ether containing a small amount of free hydrochloric acid (this *Journal*, xv, p. 155, 1913).

0.1212 gram of substance gave 0.0650 gram of H_2O and 0.2697 gram of CO_2.

	Calculated for $C_{18}H_{22}N_4O_4$.	Found.
C	60.33	60.76
H	6.14	6.01

The rotation was determined in Neuberg's pyridine-alcohol mixture in 0.5 dm. tube with D-light. 0.1000 gram of substance in 5 cc. rotated after 18 hours +0.20°.

Phenylosazone from Chondrosamine.

Three grams of chondrosamine hydrochloride were dissolved in 100 cc. of water to which the calculated amount of sodium acetate was added. The solution was warmed in the boiling water bath and to the warm solution was added the required amount of phenylhydrazine dissolved in 5 to 8 cc. of glacial acetic acid. The flask containing the solution and provided with a return condenser was heated in a boiling water bath for about three hours, and the reaction product was allowed to cool. On cooling the osazone separated in form of a flocculent mass. It was recrystallized out of hot water. The dry precipitate was washed very carefully with ether. Two recrystallizations suffice to furnish a pure, bright yellow osazone.

The melting point of the osazone dried in a vacuum desiccator over sulphuric acid was at 180–185°C.

0.1000 gram of the substance was dissolved in 5 cc. of Neuberg's pyridine-alcohol mixture and showed at pure yellow light after 18 hours' standing, a in 0.5 dm. tube at 20°, + 0.18°.

0.1280 gram substance gave 18 cc. N at 25°, 765 mm.

	Calculated.	Found.
N	15.64	15.52

Nitric Acid Oxidation of Hexosamine.

Nine grams of hexosamine hydrochloride were deaminized as in the preceding experiment with silver nitrite, the resulting solution concentrated to about 20 cc., mixed with an equal volume of concentrated nitric acid and allowed to stand over night at 42°. It was then rapidly evaporated in a shallow dish on a water bath and the syrup, after having been again evaporated with water, was diluted to 250 cc., and boiled with calcium carbonate until neutral (one-half hour). The filtrate, upon standing for two days, deposited white prisms of the calcium salt of a dibasic hexonic acid. The yield did not exceed 25 per cent of the theory. For analysis it was recrystallized by dissolving in 50 parts of boiling water containing slightly over the theoretical amount of oxalic acid and again transformed into the calcium salt by boiling with calcium carbonate. The compound contained 2 molecules of crystal water which can be removed in vacuum at 108°. By heating for sixteen hours at 138° no further appreciable loss of weight was observed.

The dried substance analyzes best for the calcium salt of a normal dibasic hexonic acid.

0.1504 gram of air-dried substance gave 0.0200 gram H_2O (108°).
0.1508 gram of air-dried substance gave 0.0208 gram H_2O (108°).
0.1509 gram of air-dried substance gave 0.0198 gram H_2O (108°).
0.1670 gram of air-dried substance gave 0.0232 gram H_2O (140°).

	Calculated for $C_6H_8O_8Ca+2H_2O$.	Found.			
		I	II	III	IV
H_2O	12.68	13.30	13.06	13.13	13.28

0.1304 gram of dried substance gave 0.0344 gram H_2O, 0.1376 gram CO_2 and 0.0302 gram CaO.

0.1312 gram of dried substance gave 0.0380 gram H_2O, 0.1380 gram CO_2 and 0.0302 gram CaO.

0.1030 gram of dried substance gave 0.0296 gram H_2O, 0.1083 gram CO_2 and 0.0243 gram CaO.

	Calculated for $C_6H_8O_8Ca$.	Found.			
		I	II	III	IV
C	29.03	28.78	28.68	28.67	
H	3.22	2.95	3.24	3.22	
CaO	22.58	23.36	21.80	23.15	22.50

0.1020 gram of substance in 2 cc. 10 per cent HCl rotated in 1 dm. tube after 10 minutes, —0.45°; after 18 hours, —0.37°.

Isosaccharic Acid Calcium from Glucosamine.—0.0786 gram of substance in 2 cc. 10 per cent HCl rotated in 1 dm. tube after 10 minutes, +0.20°; after 18 hours, +0.06°.

Brucine Salt of Hexonic Acid.

Eighteen grams of hexosamine hydrochloride were deaminized with silver nitrite and the excess of silver removed in this case with a slight excess of hydrochloric acid, and the solution (volume, 150 cc.) allowed to stand for three days with bromine added in portions so that it was always present in excess. The reaction product was after this time freed from bromine by distillation in vacuum and the hydrobromic acid removed with silver carbonate. An excess of silver in the filtrate was removed with hydrogen sulphide and the solution warmed on the water bath with 45 grams of brucine. It was then cooled and filtered from the excess of the latter and concentrated to a syrup which crystallized to a semisolid cake. This was extracted with cold alcohol, filtered, washed and recrystallized from about 100 to 150 parts of hot absolute alcohol. The first product crystallized in thin plates which are fairly easily soluble in hot alcohol, changing upon recrystallization to short heavy prisms which are then only soluble with difficulty. The product crystallized from 98 per cent alcohol seemed to contain 1 molecule of crystal water which could be removed by heating in vacuum at 100°. It analyzes best for the brucine salt of an anhydrohexonic acid.

0.1190 gram of substance gave 0.0034 gram H_2O.
0.1156 gram of substance gave 0.0640 gram H_2O and 0.2564 gram CO_2.

	Calculated for $C_{29}H_{36}N_2O_{10} + H_2O$ (590.4).	Found.
H_2O	3.11	2.86

	Calculated for $C_{29}H_{36}N_2O_{11}$ (572.4).	Found
C	60.80	60.49
H	6.10	6.25

0.3000 gram of substance in 3 cc. H_2O, weight of solution, 3.2825 grams, rotated in 0.5 dm. tube at 20° and D-light +0.77°.

$$[\alpha]_D^{20} = 16.85.$$

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, January, 1915, Vol. xx, No. 1, p. 95.]

CORRECTION.

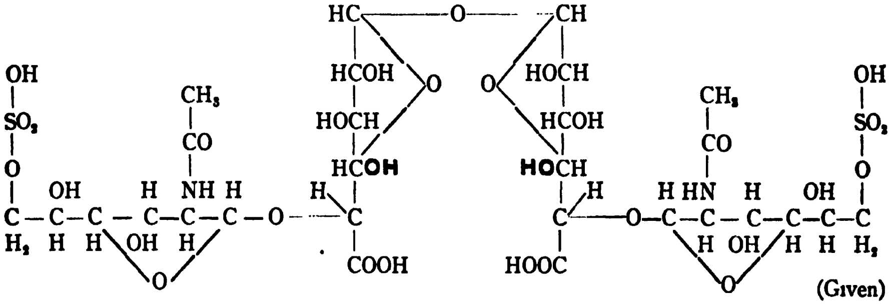
ON CHONDROITIN SULPHURIC ACID.

SECOND PAPER.[1]

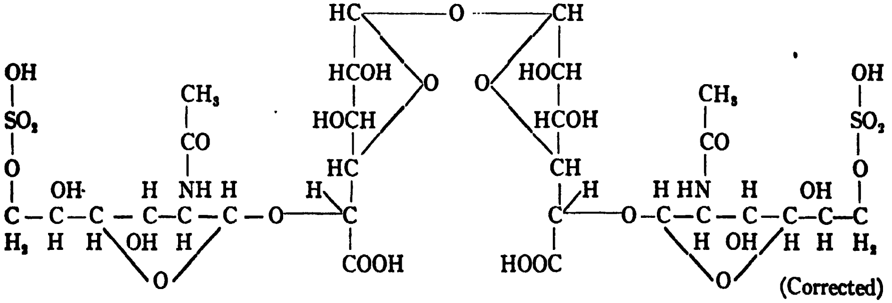
BY P. A. LEVENE AND F. B. LA FORGE.

(From The Rockefeller Institute for Medical Research, New York.)

We note with regret an error in the graphic formula of chondoitin sulphuric acid given in our second paper on this subject.



(Given)



(Corrected)

From the text of the article as well as from the formula, $C_{28}H_{44}N_2S_2O_{29}$,[2] it is evident that the error was due to an oversight and that we had no intention to alter the generally accepted view on the structure of glucosides. We are indebted to Prof. C. Neuberg for calling attention to the oversight, but we doubt that there was occasion for a "polemic." The error has no connection with the theoretical considerations expressed in the article or with the conclusions reached.

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[1] This *Journal*, xv, p. 155, 1913.

[2] This *Journal*, xviii, p. 239, 1914.

[Reprinted from The Journal of Biological Chemistry, July, 1914, Vol. xviii, No. 2, pp. 233–236.]

THE CHEMISTRY OF GLUCONEOGENESIS.

IX. The Formation of Glucose from Dioxyacetone in the Diabetic Organism.*[1]

By A. I. RINGER and E. M. FRANKEL.

(From the Department of Physiological Chemistry of the University of Pennsylvania, Philadelphia.)

The formation of glucose from dioxyacetone in the perfused liver was demonstrated recently by Embden, Schmitz and Wittenberg.[2] They found a very marked rise in the glucose concentration of the perfusion fluid after the addition of dioxyacetone.

In these experiments it was our object to study the influence of dioxyacetone on the glucose formation and acidosis in phlorhizinized dogs. We wish to state here that the plans for this work were laid about a year prior to the appearance of Embden's publication.

The dioxyacetone was kindly prepared for us by the Farbwerke-Hoechst vorm. Meister Lucius und Bruning, to whom we take pleasure in expressing our indebtedness.

The methods used were the same as those employed in experiments previously described. Because of the reducing properties of dioxyacetone, the glucose was also determined by means of the polariscopic method and the results given in the tables, so that any reduction of Fehling's solution due to dioxyacetone, which may have been secreted by the kidneys, can be detected.

The G:N ratio calculations are based upon the glucose figures obtained with Allihn's method.

In experiment XXXVIII period VI, 9.0 grams ($\frac{M}{10}$) of dioxyacetone, dissolved in 40 cc. of distilled water, were given subcutaneously. The glucose elimination, which was 7.0 grams in the

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* Received for publication, May 28, 1914.

[1] Aided by a grant from The Rockefeller Institute for Medical Research.

[2] Embden, Schmitz and Wittenberg: *Zeitschr. f. physiol. Chem.*, lxxxviii, p. 210, 1913.

fore period, rose to 13.2 and the G:N ratio, which was 3.02 in the fore period and 3.19 in the after period, rose to 5.64. If we assume that the mean ratio of 3.1 would have obtained normally in period VI, we find that 5.94 grams of "extra" glucose were eliminated in that period. In this period the acetone and acetoacetic acid were reduced from 200 mgms. to 103 mgms., to rise again to 190 in the after period.

In experiment XXXIX period VI, 9.0 grams of dioxyacetone were similarly administered subcutaneously. The glucose elimination, which was 10.9 and 11.66 grams in periods IV and V respectively, rose to 19.2 and 12.96 in periods VI and VII. The G:N ratio in periods V and VIII was 3.15 and 3.04. Assuming the mean ratio of 3.1 for periods VI and VII, we find that 9.88 grams of "extra" glucose were eliminated. In this experiment dioxyacetone exercised a fairly strong antiketogenetic effect by causing a drop in the acetone and acetoacetic acid elimination from 227 to 85 mgms., and in the β-hydroxybutyric acid elimination from 910 to 195 mgms.

In experiments XL and XLI the above results are corroborated. The "extra" glucose eliminated in experiment XL was 6.66 grams and in experiment XLI it was 5.3 grams.

In experiment XLI the total carbon[8] output in the urine was also studied. The object was to find whether any other carbonaceous

TABLE I.

Experiment XLI.

PERIOD	I CARBON IN GLUCOSE 1	II CARBON IN ACETONE AND ACETO-ACETIC ACID 2	III CARBON IN β-HYDROXY-BUTYRIC ACID 3	IV CARBON IN 1+2+3 α	V TOTAL CARBON IN URINE β	VI UNDETER-MINED CARBON β—α	REMARKS
XI	4.87	0.077	0.156	5.10	9.81	4.71	
XII	4.59	0.078	0.137	4.80	9.56	4.76	
XIII	4.98	0.075	0.144	5.20	9.93	4.73	
XIV	7.39	0.068	0.144	7.60	12.16	4.56	9.0 gms. of dioxyacetone administered containing 3.6 gms. of carbon.

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[8] The carbon was determined by the method suggested by Tangl and Kereszty: *Biochem. Zeitschr.*, xxxii, p. 266, 1911.

Experiment XXXVIII. Twelve hour periods.

DATE 1914	PERIOD	WEIGHT	NITROGEN	GLUCOSE (ALLIHN)	GLUCOSE (POLARISCOPE)	G : N	"EXTRA" GLUCOSE	ACETONE AND ACETOACETIC ACID	β-HYDROXY-BUTYRIC ACID	REMARKS
Feb.										
24	V		2.32	7.00	6.35	3.02		0.200		9 gms. of dioxyacetone dissolved in 40 cc. of water given subcut.
24	VI		2.34	13.20	13.30	5.64	5.94	0.103	0.364	
25	VII		2.17	6.94	6.45	3.19		0.190	0.775	
25	VIII		2.39	5.84	5.20	2.44		0.179	0.642	
26	IX		2.44	5.75	5.20	2.35		0.218	0.503	

Experiment XXXIX. Twelve hour periods.

DATE 1914	PERIOD	WEIGHT	NITROGEN	GLUCOSE (ALLIHN)	GLUCOSE (POLARISCOPE)	G : N	"EXTRA" GLUCOSE	ACETONE AND ACETOACETIC ACID	β-HYDROXY-BUTYRIC ACID	REMARKS
Feb.										
23	IV		3.41	10.90	10.40	3.20		0.234	0.836	
24	V	8.77	3.70	11.66	10.60	3.15		0.227	0.910	9.0 gms. of dioxyacetone as above.
24	VI		3.46	19.20	17.10	5.55	9.88	0.085	0.195	
25	VII	8.53	3.73	12.96	12.50	3.48		0.165	0.416	
25	VIII		3.42	10.40		3.04		0.150	0.435	

Experiment XL. Twelve hour periods.

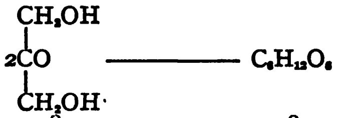
DATE 1914	PERIOD	WEIGHT	NITROGEN	GLUCOSE (ALLIHN)	GLUCOSE (POLARISCOPE)	G : N	"EXTRA" GLUCOSE	ACETONE AND ACETOACETIC ACID	β-HYDROXY-BUTYRIC ACID	REMARKS
Feb.										
3	II		3.54	10.00		2.82		0.075	0.151	9.0 gms. of dioxyacetone dissolved in 50 cc. of water given subcut.
4	III	7.20	3.15	15.70		4.99	6.66	0.046	0.081	
4	IV		3.46	10.10		2.93		0.111	0.238	

Experiment XLI. Twelve hour periods.

DATE 1914	PERIOD	WEIGHT	NITROGEN	GLUCOSE (ALLIHN)	GLUCOSE (POLARISCOPE)	G : N	"EXTRA" GLUCOSE	ACETONE AND ACETOACETIC ACID	β-HYDROXY-BUTYRIC ACID	REMARKS
May										
8	XI	11.16	4.68	12.19		2.78		0.164	0.339	
8	XII		4.39	11.48		2.62		0.167	0.296	
9	XIII		4.48	12.46	11.23	2.78		0.160	0.312	9.0 gms. of dioxyacetone as above.
9	XIV		4.72	18.46	17.65	3.92	5.3	0.145	0.312	
10	XV	10.82				3.20		0.155	0.269	
10	XVI		4.28	12.67	11.86	2.96		0.126	0.273	

material, not utilized in the body, was eliminated as a result of the dioxyacetone administration. The carbon of the glucose, acetoacetic acid, β-hydroxybutyric acid was calculated from the figures obtained in their determinations. The sum of these, subtracted from the total carbon, gives the undetermined carbon fraction which would rise if any other product of dioxyacetone metabolism found its way into the urine.

Table I gives the results of this investigation. Column VI gives the values of the undetermined carbon. From the fact that there was no increase in that fraction (period XIV) when dioxyacetone was administered, we feel justified in assuming that all of the dioxyacetone is either converted into glucose or burned. That the conversion of dioxyacetone into glucose may be quantitative is evident from experiment XXXIV.



$$2\begin{array}{l} CH_2OH \\ | \\ CO \\ | \\ CH_2OH \end{array} \longrightarrow C_6H_{12}O_6$$

$$180 = 180$$

$$9 = 9 \quad \text{(found 9.88)}$$

Miller and Taylor[4] recently found that dioxyacetone, in acid solution, acts as a very strong reducing agent of ammonium molybdate. This fact was utilized in detecting its presence in the urine in unchanged form. In experiment XL it was strongly positive; the urine also reduced Fehling's solution in the cold. In none of the other experiments was this observed.

SUMMARY AND CONCLUSIONS.

Four experiments were performed in which dioxyacetone was administered subcutaneously to phlorhizinized dogs. In every case a rise in the glucose elimination followed. The "extra" glucose in one experiment corresponded to an amount of glucose which would arise if all of the carbon of dioxyacetone were converted into glucose.

The effect of dioxyacetone on acidosis is decidedly antiketogenetic in three of the four experiments.

Dioxyacetone was found in the urine in unchanged form in only one of the four experiments. In that case the urine reduced Fehling's solution in the cold and reacted positively with the Miller-Taylor reagent. In none of the other experiments did dioxyacetone appear in the urine.

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[4] Miller and Taylor: this *Journal*, xvii, p. 531, 1914.

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, July, 1914, Vol. xviii, No. 2, pp. 237-240.]

ON THE CONJUGATED SULPHURIC ACID FROM TENDOMUCOID.*

BY P. A. LEVENE AND F. B. LA FORGE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The presence of a conjugated sulphuric acid in the molecule of tendomucoid was first discovered by Levene.[1] Later J. A. Mandel and Levene[2] have shown the presence of a similar acid in many tissues and even in leucocytes. All these new conjugated sulphuric acids resembled in their properties the chondroitin sulphuric acid obtained from cartilage by Mörner[3] and by Schmiedeberg.[4] The exact relationship of these various acids to one another and of all of them towards chondroitin sulphuric acid could not be established with certainty, since the details of the structure of the substance of Mörner and Schmiedeberg had not been known. Provisionally all conjugated sulphuric acids derived from any other source than from cartilage were named glycothionic acids.

In course of the last two years, through the work of the present writers,[5] our knowledge of the structure of chondroitin sulphuric acid has made considerable progress. All the components of the substance have been isolated and the mode of their union in a great measure was elucidated. One component of chondroitin sulphuric acid was found to be of special significance, namely the amino hexose. This proved to be a new nitrogenous hexose—chondrosamine—quite distinct from glucosamine. The complex of this hexose with glucuronic acid—chondrosin—is also a very definite

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* Received for publication, May 28, 1914.

[1] Levene: *Zeitschr. f. physiol. Chem.*, xxxi, p. 395, 1901.

[2] J. A. Mandel and Levene: *Zeitschr. f. physiol. Chem.*, xlv, p. 386, 1905; *Biochem. Zeitschr.*, iv, p. 78, 1907.

[3] Mörner: *Skand. Arch. f. Physiol.*, i, p. 210, 1889.

[4] Schmiedeberg: *Arch. f. exp. Path. u. Pharm.*, xxviii, p. 358, 1903.

[5] Levene and La Forge: this *Journal*, xv, pp. 69 and 155, 1913; xviii, p. 123, 1914.

component of chondroitin sulphuric acid. Further, the ratio of nitrogen to carbon, 1 : 14, was found quite characteristic for chondroitin sulphuric acid. Finally, the presence of one acetyl group in the molecule may be regarded as typical of the same acid.

The analysis of the conjugated sulphuric acid obtained from tendomucoid resulted in the discovery that the components of this acid were identical with those of chondroitin sulphuric acid: they were the same hexosamine, chondrosamine, the same glucuronic acid, besides acetic and sulphuric acids. Similarly to chondroitin sulphuric acid the substance yielded, on hydrolysis with hydrochloric acid, chondrosin hydrochloride. The ratio of nitrogen to carbon was 1 : 14, as in chondroitin sulphuric acid. The substance similarly to chondroitin sulphuric acid also contained one acetyl group in its molecule.

Hence one is forced to accept the identity of the conjugated sulphuric acid from tendomucoid with that of chondroitin sulphuric acid from cartilage.

EXPERIMENTAL.

Preparation of Tendomucoid and Chondroitin Sulphuric Acid.

Owing to the nature of the combined protein the method of preparing chondroitin sulphuric acid from tendons differs somewhat from its preparation from cartilage. Portions of 50 achilles tendons from cattle were cleaned, passed through a hashing machine and allowed to stand over night with 20 liters of two-thirds saturated lime water. The liquid was strained off and the process repeated once again on the residue. The combined filtrates were just acidified with hydrochloric acid which produced a flocculent precipitate of tendomucoid. The supernatant liquid was then siphoned off and after addition of an equal volume of 95 per cent alcohol the mucoid was filtered off on a folded filter. The moist product was agitated for some time with 1.5 liters of a 2 per cent potassium hydrate solution. After standing over night the turbid brown solution was acidified with acetic acid and the separated protein removed by filtration on a folded filter. The filtrates from two such experiments were neutralized with sodium hydrate and the chondroitin sulphuric acid precipitated by a solution of basic lead acetate. The lead precipitate was repeatedly washed by triturating in a mortar with distilled water and filtering with suction. The washed product was suspended in about 2 liters of water; 10 cc. of glacial acetic acid and 20 grams of barium acetate were added and decomposition effected by passing in hydrogen sulphide with constant stirring. The lead sulphide was filtered off with suction, the filtrate concentrated to about 350 cc., and the barium salt precipitated by the addition of about 250 cc. of alcohol. It was then filtered with suction, washed, first with 50 per cent, then with 95 per cent, and finally with absolute alcohol and ether. The yield amounts to about 12 to 15 grams.

0.2220 gram of substance gave 0.2043 gram CO_2 and 0.0775 gram H_2O.

0.6136 gram of substance gave 9.25 cc. $\frac{N}{10}$ NH_3 (Kjeldahl).

0.6141 gram of substance gave 9.50 cc. $\frac{N}{10}$ acetic acid.

0.7002 gram of substance gave 0.2170 gram $BaSO_4$.

	Calculated for $C_{28}H_{44}N_2S_2O_{28}$.	Found.
C	27.80	25.13
H	3.48	3.88
N	2.32	2.11
S	5.30	4.26
Ba	22.70	18.35

N : C = 1 : 13.89

Chondrosin hydrochloride was prepared exactly as described in a previous communication.[6]

0.1258 gram of substance gave 0.1653 gram CO_2 and 0.0685 gram H_2O.

0.2238 gram of substance gave 13.6 cc. amino N at 20°, 763 mm.

0.4024 gram of substance in 3 cc. of water, weight of solution 3.3792 grams, rotated in a 0.5 dm. tube at 20° with D-light $+2.46°$.

Chondrosin Hydrochloride from Cartilage.

0.1584 gram of substance gave 0.2167 gram CO_2 and 0.0806 gram H_2O.

0.2085 gram of substance gave 12.5 cc. amino N at 18°, 765 mm.

0.6543 gram of substance in 3 cc. of water, weight of solution 3.6481 grams, rotated in a 0.5 dm. tube with D-light $+ 3.90°$.

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[6] This *Journal*, xv, p. 73, 1913.

	Calculated for $C_{12}H_{21}NO_{11}HCl$ (390.5).	Found. From tendons.	Found. From cartilage.
C	36.9	35.8	37.3
H	5.64	6.0	5.66
N	3.58	3.45	3.45
N:C=		1:12.1	1:12.6
$[\alpha]_D^{20}$		+41.5	+43.4

The hexosamine hydrochloride was prepared in exactly the same manner as already described.[7] From 18 grams of the barium salt 3.5 grams amino hexose were obtained. For analysis it was dissolved in 3 parts of water with the addition of a few drops of hydrochloric acid, allowed to crystallize by evaporation and dried in a desiccator. M.P., 180°.

0.1596 gram of substance gave 0.1930 gram CO_2 and 0.0945 gram H_2O.

0.1516 gram of substance gave 17.6 cc. amino N at 19°, 774 mm.

0.1932 gram of substance in 2 cc. of water, weight of solution 2,1904 grams, rotated in a 1 dm. tube at 20° with D-light:

After about fifteen minutes	+10.75°
After twenty-four hours	+ 8.5°

	Calculated for $C_6H_{13}O_5NHCl$.	Found.
C	33.40	33.01
H	6.54	6.57
N	6.51	6.79

$[\alpha]_D^{20}$ (for equilibrium condition without consideration of specific gravity) $= +96.4°$.

Glucuronic acid osazone hydrazid was prepared exactly as previously described.[8] From 4 grams of chondrosin hydrochloride 0.1 gram of the substance was obtained; M.P. exactly the same as the product from glucuronic acid cartilage chondrosin, 122°.[9]

0.0805 gram of substance gave 12.1 cc. N (Dumas) at 24°, 766 mm.

	Calculated for $C_{24}H_{26}N_6O_4 + 1.5\,H_2O$.	Found.
N	17.17	16.93

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[7] This *Journal*, xv, p. 158, 1913; xviii, p. 123, 1914.

[8] This *Journal*, xv, p. 75, 1913.

[9] This melting point, owing to a typographical error, was reported 115° (this *Journal*, xv, p. 75). The correct melting point is 122° with decomposition.

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ON VICINE.*

BY P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Vicine was discovered by Ritthausen[1] who found that the substance could be hydrolyzed into a hexose and into a basic substance which was named by him divicine. The exact nature of the base and the configuration of the sugar were not recognized by the discoverer of the substance. Schulze and Trier,[2] on the basis of theoretical considerations, were the first to give expression to the assumption that vicine had the structure of a pyrimidine glucoside. Very recently T. B. Johnson,[3] and Johnson and Johns[4] have added both theoretical and experimental evidence in support of the same assumption. These authors, following the directions of Traube, prepared two pyrimidine bases: 4-6-dioxy-2-5-diamino-pyrimidine and 2-6-dioxy-4-5-diamino-pyrimidine. Comparing the properties of the two synthetic bases with the properties of divicine as described by Ritthausen the authors were led to the belief that divicine was identical with 2-6-dioxy-4-5-diamino-pyrimidine.

At the time of the publication of T. B. Johnson's first article the present work was already in progress, and we then entered into an agreement with Professor Johnson to postpone further experiments until after the publication of the further two articles by Johnson and Johns. Since the articles of Johnson and Johns do not exhaust the subject of the structure of vicine, I feel justified in publishing the results of the present investigation, which furnish

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* Received for publication, June 2, 1914.

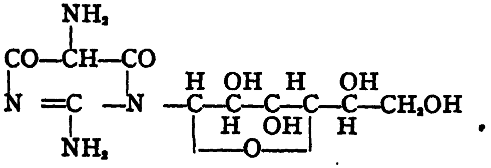
[1] Ritthausen: *Journ. f. prakt. Chem.*, ii, p. 336, 1870; vii, p. 374, 1873; *Ber. d. deutsch. chem. Gesellsch.*, ix, p. 301, 1876; *Journ. f. prakt. Chem.*, xxiv, p. 202, 1881; *Ber. d. deutsch. chem. Gesellsch.*, xxix, pp. 894 and 2108, 1896; *Journ. f. prakt. Chem.*, lix, pp. 480 and 487, 1899.

[2] Schulze and Trier: *Zeitschr. f. physiol. Chem.*, lxx, p. 143, 1910.

[3] Johnson: *Journ. Amer. Chem. Soc.*, xxxvi, p. 337, 1914.

[4] Johnson and Johns: *Journ. Amer. Chem. Soc.*, xxxvi, pp. 545 and 970, 1914.

information on the principal points in the structure of the nucleoside. The results point to the conclusion that vicine is composed of 4-6-dioxy-2-5-diamino-pyrimidine combined in glycosidic union with *d*-glucose. Regarding the place of the union between sugar and base the information is not yet absolutely definite. The fact that both vicine and divicine seem to give off about 50 per cent of their nitrogen in form of nitrogen gas on treatment with nitrous acid seems to indicate that the union is not through the medium of one of the amino-groups. Hence one may be justified in representing vicine provisionally by the following graphic formula:



This conception, however, is not in complete harmony with all of its known properties.

It seems to be contradicted by the fact that vicine does not give the color test with molybdic acid. Divicine, on the other hand, does give a positive color test with the same reagent, and Johnson and Johns reached the conclusion that the presence of a free amino-group in position 5 results in a positive molybdic test.

The nature of the sugar is based on properties of the osazone: M.P. = 205°C. and rotation in a 0.5 dm. tube (0.200 gram in 10 cc. of Neuberg's pyridine-alcohol mixture) was: Initial, —0.49°; equilibrium, —0.39°. The rotation of a glucosazone under the same condition was identical. On oxidation with nitric acid the sugar formed saccharic acid, which was identified as the acid potassium salt.

The base was recognized as 4-6-dioxy-2-5-diamino-pyrimidine on the following grounds. The 4-6-dioxy-2-5-diamino and 2-6-dioxy-4-5-diamino-pyrimidines were prepared by the method of Traube. These two bases differ one from another in the following three properties. The first base forms a sulphate which crystallizes without or with one molecule of water of crystallization, on treatment with nitrous acid in the Van Slyke apparatus it gives rise to nitrogen gas in a quantity equal to about 80 per cent of that required for two primary amino-groups, and on condensation with urea, under the conditions given by Johnson and Johns, it does not give rise to uric acid. The second base forms a sulphate, crystallizing out of water with one and one-half molecules of water according to Traube; the sulphate prepared in course of this work contained about two and one-half molecules of water; with nitrous acid in the apparatus of Van Slyke it forms a very insignificant quantity of nitrogen gas (perhaps due to impurity). It is interesting to note that 2-6-dioxy-4-amino-pyrimidine under the same conditions forms nitrogen gas in quantity equal to 33 per cent of its total nitrogen. Further, as shown by Johnson and Johns, the base condenses with urea to form uric acid.

Divicine, in most of its properties, comes closer to 4-6-dioxy-2-5-diamino-pyrimidine than to its isomer. True, it was difficult to obtain uniform analytical figures for the sulphate of divicine, a fact already observed by Ritthausen, but of many samples analyzed all possessed more resemblance to the first of the two bases. In its behavior towards nitrous acid and towards urea it was decidedly different from 2-6-dioxy-4-5-diamino-pyrimidine, on the contrary it showed in this respect a striking resemblance to the isomer.

EXPERIMENTAL PART.

Preparation of vicine was facilitated by slight modifications in the original Ritthausen method. The vetch meal was extracted with 5 per cent sulphuric acid for three hours, at the end of which the mixture was neutralized with barium hydrate solution, filtered, and the filtrate precipitated with a 10 per cent solution of mercuric sulphate in 5 per cent sulphuric acid. The mixture again was neutralized with barium hydrate solution. The precipitate was allowed to settle and was then removed by filtration, washed and suspended in water to which an excess of barium carbonate was added. The precipitate was finally freed from mercury by means of hydrogen sulphide and the filtrate from mercuric sulphide concentrated to a small volume, first on the water bath and finally under diminished pressure. To the concentrated solution 95 per cent alcohol was added as long as it formed a precipitate and the mixture was brought to a boil on water bath. It was then filtered, and the filtrate concentrated to a very small volume under diminished pressure. Analytically pure and colorless vicine crystallized out on cooling. Once recrystallized the substance had a M.P. = 242°C. (uncorrected) and the following optical rotation:

0.300 gram of substance in 3 cc. of water had the weight of 3.7934 grams. The rotation in 1 dm. tube in pure yellow light was $\alpha = -0.93°$. Hence

$$[\alpha]_D^{25} = -11.7°$$

Nitrogen Distribution in Vicine.

0.1000 gram of the substance contained 0.01736 gram of nitrogen.

0.1000 gram of a second sample contained 0.0174 gram of nitrogen.

Amino Nitrogen Estimation.—0.050 gram was used in the first experiment. The conditions of experiment were the usual indicated by Van Slyke. In forty minutes the evolution of nitrogen reached its maximum. It was 8 cc. of gas at 30°C., 769 mm., or amino N = 50 per cent of the total nitrogen.

It was thought that the high amino-nitrogen value was conditioned by the fact that the nucleoside was hydrolyzed in course of the experiment. Hence the acidity of the nitrite mixture was diminished by employing one part of acetic acid to 5 parts of the sodium nitrite solution.

0.050 gram of the vicine evolved the following values of nitrogen gas:

15 minutes	5.4 cc.
25 minutes	8.8 cc.
40 minutes. (No further changes)	8.8 cc.

Correction, 0.8 cc. Hence final volume = 8 cc. of N at 26°C. and 760 mm., or amino N = 49.98 per cent of total nitrogen.

It is realized that other methods will have to be employed to determine the number of free NH_2 groups in the vicine molecule.

Preparation of Divicine Sulphate and Its Composition.—Five-gram lots of vicine were taken up in 25 cc. of a 20 per cent solution of sulphuric acid and placed in a boiling water bath. A crystalline deposit soon appeared in the solution. As soon as the deposit reached its maximum the heating was discontinued, the reaction product cooled and the precipitate filtered and dried in the air over night. Before analysis the substance was recrystallized out of 10 per cent sulphuric acid.

0.1176 gram of the substance gave 0.1066 gram CO_2 and 0.4100 gram H_2O.

0.1000 gram of the substance gave 0.0374 gram $BaSO_4$.

0.050 gram of the substance gave 0.0139 gram nitrogen by Kjeldahl method.

0.050 gram of the substance dissolved in 5 cc. of 10 per cent solution of sulphuric acid gave 0.0064 gram amino N in the Van Slyke apparatus.

Analysis of the Sulphate of 2-6-Dioxy-4-5-Diamino-Pyrimidine. —The substance was prepared according to the method of Traube.

Sample I.—0.1181 gram of substance gave 0.0979 gram CO_2 and 0.0466 gram H_2O.

0.1000 gram of substance gave 0.0518 gram $BaSO_4$.

0.050 gram of substance gave 0.1345 gram nitrogen by Kjeldahl method.

0.050 gram of substance dissolved in 10 per cent solution of sulphuric acid gave 0.0007 gram nitrogen by Van Slyke method.

Sample II.—0.050 gram of substance gave 0.0135 gram nitrogen by Kjeldahl method.

0.050 gram of substance dissolved in a 10 per cent solution of sulphuric acid gave 0.0009 gram nitrogen in Van Slyke apparatus.

Analysis of 2-6-Dioxy-4-Amino-Pyrimidine.

0.050 gram of substance gave 0.0165 gram nitrogen by Kjeldahl method.

0.050 gram of substance dissolved in a 10 per cent solution of sulphuric acid gave by Van Slyke method 0.0051 gram of nitrogen. Hence, ratio of $\frac{\text{Amino N}}{\text{Total N}} = 1:3$.

Analysis of 4-6-Dioxy-2-5-Diamino-Pyrimidine.—The base was prepared by the two methods of Traube. The first sample by reducing with a current of hydrogen sulphide the isonitrosomalonylguanidine in hydrochloric acid solution. The hydrochloride of the base was then transformed into the sulphate. The hydrochloride was employed for the estimation of the amino-nitrogen since the sulphate was found too insoluble for the purpose.

The second sample of the base was prepared by the sulphuric acid process, in which the isolation of the isonitrosoderivative is omitted.

The composition of the sulphates obtained by the two different processes was identical.

0.050 gram of the hydrochloride gave 0.01414 gram of nitrogen by the Kjeldahl method.

0.050 gram of the same substance gave 0.00505 gram of nitrogen in Van Slyke's apparatus.

0.050 gram of the sulphate obtained from the hydrochloride gave 0.01335 gram of nitrogen by the Kjeldahl method.

0.100 gram of the substance gave 0.0582 gram $BaSO_4$.

0.050 gram of the second sample of the sulphate gave 0.0142 gram of nitrogen by the Kjeldahl method.

0.100 gram of the substance gave 0.0598 gram of $BaSO_4$.

Analysis of results.

	Calculated for 2-6-dioxy-4-5-diamino-pyrimidine $(C_4H_6N_4O_2)_2H_2SO_4 + 2\frac{1}{2}H_2O$.	Found.
C	22.50	22.61
H	4.50	4.42
N	27.00	(I) 26.90 (II) 27.00
NH_2N	13.50	(trace)
H_2SO_4	22.90	22.11

	Calculated for 4-6-dioxy-2-5-diamine $(C_4H_6N_4O_2)_2H_2SO_4 + H_2O$.	Found.
N	28.00	(I) 27.80 (II) 27.70
H_2SO_4	24.50	(I) 24.28 (II) 24.45

	Calculated for $(C_4H_6N_4O_2)HCl + H_2O$.	Found.
N	28.49	28.28
NH_2N	14.24	11.30

	Calculated for Divicine Sulphate $(C_4H_6N_4O_2)H_2SO_4 + H_2O$.	Found.
C	24.00	24.72
H	4.00	3.90
N	28.00	27.80
NH_2N	14.00	12.80
H_2SO_4	24.50	24.16

Condensation with Urea.

2-6-Dioxy-4-5-Diamino-Pyrimidine.—0.5 gram of the free base and 0.5 gram of urea were heated in a small tube at 160 to 170°C. for one hour. The reaction product was dissolved by the aid of very little alkali, and acidulated by means of hydrochloric acid. A very light yellow precipitate formed, which was carefully washed and dried. With ammoniacal silver solution it formed a precipitate insoluble in excess of ammonia.

0.050 gram of the substance gave 0.0165 gram nitrogen by Kjeldahl method.

	Calculated for $C_5H_4N_4O_3$.	Found.
N	33.33	33.00

In a second experiment 1 gram of the sulphate was intimately triturated with 1.5 grams of urea and treated as in the first experiment. The final product had the same appearance as in the first experiment.

0.050 gram of the substance gave 0.01659 gram nitrogen by Kjeldahl method.

	Calculated for $C_5H_4N_4O_3$.	Found.
N	33.33	33.18

4-6-dioxy-2-5-diamino-pyrimidine, and divicine on condensation with urea under identical conditions did not form uric acid.

Analysis of Carbohydrate.—10 grams of vicine were hydrolyzed in the usual way. The divicine sulphate was removed by filtration. The filtrate diluted to 300 cc. and the sulphuric acid removed by means of lead carbonate, and the lead by means of hydrogen sulphide. A part of the solution was used for the preparation of the phenyl osazone. It was prepared in the usual way. For analysis it was recrystallized out of an alcohol-acetone mixture. The melting point = 205°C.

0.1187 gram of the substance gave 0.2635 gram CO_2 and 0.0652 gram H_2O.

	Calculated for $C_{18}H_{22}N_4O_4$.	Found.
C	60.30	60.54
H	6.14	6.15

0.200 gram of the substance dissolved in a mixture of 2 cc. pyridine and 3 cc. of alcohol rotated in a 0.5 dm. tube in yellow light, $\alpha = -0.49°$; in twenty-four hours, $\alpha = -0.39°$.

0.200 gram of glucosazone under the same condition, $\alpha = -0.49°$; in twenty-four hours, $\alpha = -0.39°$.

The remaining part of the solution was oxidized with nitric acid and from the reaction product the acid potassium salt of saccharic acid obtained. For analysis it was once recrystallized out of water.

0.100 gram of the substance gave 0.0354 gram K_2SO_4.

	Calculated for $C_6H_9O_8K$.	Found.
K	15.74	15.90

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NOTE ON A CASE OF PENTOSURIA.

Second Communication.*

By P. A. LEVENE and F. B. La FORGE.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

In a previous communication[1] we reported some observations on the nature of the pentose in the urine of a person apparently in good health. At the time of the first communication only one derivative of the sugar had been obtained, namely its phenylosazone. Since then the method for obtaining a concentrated solution of the sugar has been improved. This permitted the preparation of the *p*-bromphenylhydrazone. The hydrazone was converted into the free sugar. However, as yet it was not possible to obtain it crystalline. Hence the conclusions regarding the nature of the sugar have to be based on indirect evidence.

At present there exist the following data: first, on the properties of the phenylosazone; second, on the *p*-bromphenylosazone; third, on the optical rotation of the free sugar. The osazone had a melting point of 160 to 163°C., an initial optical rotation of $\alpha = +0.15°$, and equilibrium of $\alpha = +0.57°$ (conditions given in experimental part). The osazone mixed with that of *l*-arabinose melted at 169–178°C., whereas a mixture of equal parts of the urine osazone and of *l*-xylose melted at 203°C.

The *p*-bromphenylhydrazone had a melting point of 130–131°C., and mixed with *l*-xylose-*p*-bromphenylhydrazone, 121–122°C. The rotation of the hydrazone was: initial $\alpha = -0.70°$, equilibrium $\alpha = +1.06°$ (the conditions are given in the experimental part).

The rotation of the sugar was approximately $[\alpha]_D^{20} = +33.1°$.

Discussion of the Data.—The properties of the osazone permit

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* Received for publication, June 5, 1914.

[1] Levene and La Forge: this *Journal*, xv, p. 481, 1913.

the conclusion that the urine sugar belongs to the xylose group on the following grounds:

First, it was found by Fischer,[2] and recently pointed out by Zerner and Waltuch,[3] that *dl*-xylosazone has a melting point = 205°C., whereas the melting point of *dl*-arabinosazone is only slightly above that of the optically active osazones. Zerner and Waltuch suggested a method for diagnosing a pentose by observing the change in the melting point of its osazone produced by addition of either xylosazone or of arabinosazone of the opposite optical rotation. On the basis of this, a dextrorotatory pentosazone, which shows an elevation of its melting point after being mixed with a levorotatory xylosazone from 163° to 205°C., has to be regarded as xylosazone. The urine pentosazone acted in this manner, and hence its structure is that of a xylosazone.

This conclusion is further substantiated by the character of its mutarotation. It was found in course of this work that the initial optical rotation of xylosazone is lower than the equilibrium rotation, whereas the reverse is true for arabinosazone.

The optical rotation of the urine osazone increases in magnitude on standing. This again is good evidence in support of the xylosazone nature of the urine pentosazone.

A dextrorotatory xylosazone may be derived from one of the following three sugars: from *d*-xylose (according to Fischer's nomenclature), from *l*-lyxose, or from the ketopentose corresponding to these two aldoses. The urine pentose is dextrorotatory, hence it cannot be *d*-xylose, for this is levorotatory. It also differs from *d*-xylose by the properties of its *p*-bromphenylhydrazone. The optical rotation of the two substances is markedly different. Their melting points seem to be identical, but the mixed melting point of the two substances shows an unmistakable depression from that of either one.

On the basis of all these considerations the urine pentose cannot be regarded as xylose.

There remain only two other possible explanations of the structure of the urine pentose: It may be regarded either as *l*-lyxose or *l*-ketolyxose (*d*-ketoxylose).

[2] Fischer: *Ber. d. deutsch. chem. Gesellsch.*, xxvii, p. 2486, 1894.

[3] Zerner and Waltuch: *Monatsch. f. Chem.*, xxxiv, p. 1639, 1913.

The first assumption is readily removed by a consideration of the properties of the *p*-bromphenylhydrazones. There is a difference of 30° in their respective melting points. The lyxose derivative is levorotatory, and the range of its mutarotation is smaller than that of the derivative of the urine pentose. Hence also the lyxose possibility has to be abandoned.

By exclusion there remains the last possibility, namely that the urine pentose has the structure of a ketopentose. This assumption in a measure is substantiated by oxidation experiments. By means of nitric acid it was not possible to obtain trioxyglutaric acid. Bromine remained practically without influence on the urine pentose under the same conditions, which brought about a complete oxidation of xylose to xylonic acid. It is noteworthy that fructose is also not attacked by bromine under conditions suitable for oxidation of glucose to gluconic acid.

Hence, all present evidence seems to support the view that the urine pentose analyzed in this work is a ketopentose corresponding to *l*-lyxose or *d*-xylose (Fischer's nomenclature). We expect to continue the work on the products of nitric acid oxidation, and we also expect to prepare the sugar synthetically.

We wish to mention that the sugar on distillation with hydrochloric acid formed very considerable quantities of furfurols, although the yields of this substance were variable.

In conclusion we wish to state that we excluded the possible objection that the ketosugar was produced artificially by an intramolecular rearrangement brought about by the action of lead and barium. A solution of xylose was treated with lead and barium in exactly the same manner as the urine. The lead precipitate of the pentose was freed from lead. It was found that the recovered sugar had the same properties as the original xylose.

EXPERIMENTAL PART.

The urine pentosazone has been prepared directly from the urine[4] and also from the purified sugar solution. All other osazones used in these experiments were prepared from the pure sugars by heating with phenylhydrazine and acetic acid in the usual manner. *d*-Arabinosazone was obtained from *d*-ribose. All determinations were made by using 0.1 gram substance in 5 cc. pyridine-alcohol mixture with D-light in 0.5 dm. tube.

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[4] This *Journal*, xv, p. 484, 1913.

d-Xylosazone. I. Soon after preparation of solution $[\alpha]_D = -0.10$
After about eighteen hours.............................. -0.36
II. The same ten minutes after preparation of solution.... -0.09
After one hour.. -0.21
After twenty-four hours................................. -0.43
l-Arabinosazone. Soon after preparation of solution $[\alpha]_D = +0.55$
After about eighteen hours.............................. $+0.30$
d-Arabinosazone. Soon after preparation of solution $[\alpha]_D = -0.50$
After about eighteen hours.............................. -0.33
Osazone of the urine pentose. I. Soon after preparation of solution $[\alpha]_D$ .... $= +0.15$
After five hours.. $+0.34$
After eight hours.. $+0.47$
II. About fifteen minutes after preparation of solution...... $+0.27$
After eighteen hours.. $+0.57$

Melting Points of Osazones.

OSAZONES	CONTRACTED AT °C.	MELTED AT °C.	EFFERVESCED AT °C.
l-Arabinose	160	166	200
d- + *l*-Arabinose	160	170	200
l-Arabinose + *d*-xylose	157	163	180
d-Xylose		164	167
Urine pentose	155	160	163
l-Xylose + urine pentose	194	201	203
Urine pentose + *l*-arabinose	159	169	178

Pentose-p-Bromphenylhydrazone.

Fifteen liters of pentose urine were allowed to stand for twelve hours at room temperature with about 75 grams of soy bean meal, during which time a slow stream of carbon dioxide was allowed to bubble through the liquid. Then, without filtering, a warm concentrated solution of barium acetate was added as long as a precipitate continued to be formed. This was filtered off and washed thoroughly with water, the washings being added to the filtrate which was then precipitated with a concentrated solution of lead acetate. The rather large deposit of lead salts was twice washed by triturating in a mortar with water and filtering on a Buchner funnel. The precipitate was then rejected and the combined filtrates which had a volume of about 20 to 25 liters were treated with a solution of basic lead acetate which produces a voluminous white precipitate containing together with other substances practically all the pentose. At least three washings are necessary to remove sufficiently the adherent solution from the precipitate, after which it was suspended in about 3 liters of water and while being vigorously agitated with a turbine a very rapid stream of carbon dioxide was passed through the suspension. By this treatment the total amount of the pentose together with some other substances are liberated from their metal compounds and pass into solution, while many impurities remain behind in the precipitate. About three-quarters of an hour is sufficient for the operation. The bulky residue must be extracted three times with about 1.5 liters of water to remove all the adhering pentose. The combined filtrates and washings, if their volumes exceeded 5 liters, were concentrated under diminished pressure to that volume, and substances other than pentose present as lead salts transformed into barium salts by addition of hot concentrated barium hydrate solution to distinct alkaline reaction and neutralizing immediately by passing in carbon dioxide. Lead carbonate was removed by filtration. The filtrate was concentrated under diminished pressure at as low a temperature as possible to about 75 cc., and then slowly dropped into 1.5 liters of 100 per cent alcohol, kept agitated with a turbine. One hundred and fifty cc. of dry ether were then added and the voluminous precipitate which settled readily after a few minutes was filtered off with suction. The yield of the pentose was increased somewhat by grinding the barium salts in a mortar with 300 cc. of 100 per cent alcohol, allowing it to stand over night, after which the filtrate was combined with the first.

The alcoholic solution of the pentose was concentrated in vacuum to about 45–50 cc. and the precipitation with alcohol and ether repeated. After filtering from the barium salts as before, the filtrate was concentrated in vacuum to about 75–80 cc.,[5] transferred to a glass dish and the calculated amount of parabromphenylhydrazine added. It was then heated for about ten minutes on the water bath, stirred until the hydrazine was dissolved, allowed to cool and then placed in a vacuum desiccator over solid potassium hydrate. Ordinarily crystallization begins in a few minutes and after about twelve hours practically all of the hydrazone has crystallized to a semisolid cake. It often happened, however, that some was left in the form of a thick light-brown syrup. In either case, addition of 75 to 100 cc. of cold water causes complete crystallization. After about two hours' standing in the refrigerator the hydrazone was filtered off and washed with a little cold water and then without drying extracted with ether as long as colored material was removed. The crude product was recrystallized by dissolving in about 2–2½ parts of alcohol (filtering if necessary) and diluting with 4–5 parts of cold water. Soon the hydrazone began to separate in pale yellow plates or scales, which after a time filled the whole volume of the liquid. There is very little loss of material involved in the purification and the yield of the final product which had been washed with ether and dried was about 15 grams, representing about 40 per cent of the total pentose present in the urine.

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[5] A slight turbidity or even a light precipitate at this stage is of no significance and may be disregarded. It is most convenient to determine the amount of pentose before the solution has been concentrated below 500 cc.

The hydrazone melted at 128–129° (corr. 130–131°) and decomposed at 154°. The melting point remained unchanged on further recrystallization. A mixed melting point determination with parabromphenylhydrazone of xylose showed a depression of 8° (M.P., 121°; corr. 122°).

0.1265 gram of substance gave 0.1920 gram CO_2 and 0.0538 gram H_2O.

	Calculated for $C_{11}H_{15}N_2BrO_4$.	Found.
C	41.39	41.43
H	4.71	4.76

I. 0.3000 gram of substance in 3 cc. of alcohol rotated in 0.5 dm. tube, with D-light:

After five minutes	−1.00°
After fifteen minutes	−0.93°
After five hours	+1.12°
After eighteen hours	+1.12°

II. 0.2000 gram of substance in 3 cc. of alcohol rotated in 0.5 dm. tube, with D-light:

After ten minutes	−0.70°
After forty minutes	−0.55°
After ninety minutes	−0.25°

After three hours	+0.25°
After five hours	+0.72°
After thirty-six hours	+0.82°

0.2000 gram of substance in 3 cc. pyridine rotated in 0.5 dm. tube with D-light:

After ten minutes	—0.87°
After two hours	—0.86°
After thirty-six hours	+1.06°

The barium salts, above referred to, obtained from the alcoholic precipitation have not been thoroughly investigated. They were for the most part crystalline and showed a slight optical activity which might have been due to the presence of some compound of the pentose. They analyzed very close to barium acetate.

	Calculated for $Ba(CH_3COO)_2$.	Found.
C	18.8	17.9
H	2.3	2.9
Ba	53.6	45.1

0.2022 gram of substance in 2 cc. water rotated in 0.5 dm. tube with D-light, + 0.26°.

Rotation of l-Xylose-Parabromphenylhydrazine in Pyridine and in Alcohol.

I. 0.3000 gram of substance in 3 cc. alcohol rotated in 0.5 dm. tube with D-light, + 0.06°, and remained unchanged for forty-eight hours.

II. 0.3000 gram of substance in 3 cc. of alcohol + a few drops of pyridine rotated in 0.5 dm. tube with D-light, — 0.06°; after eighteen hours, + 0.06°.

III. 0.3000 gram of substance in 3 cc. pyridine rotated in 0.5 dm. tube with D-light:

After ten minutes	—1.22°
After eighteen hours	—1.00°
After forty-eight hours	—0.42°

Parabrom- and Paranitrophenylhydrazones of d-Lyxose.

Both of these derivatives possess better properties than the benzylphenylhydrazone described by Ruff.[6] They were prepared by bringing together the calculated amounts of *d*-lyxose and the respective hydrazines in 75 per cent alcoholic solution. Both were recrystallized from 95 per cent alcohol.

d-Lyxose-*p*-bromphenylhydrazone melted at 158° (corr. 161.5°).

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[6] Ruff: *Ber. d. deutsch. chem. Gesellsch.*, xxxii, p. 552, 1899; Ruff and Ollendorff: *ibid.*, xxxiii, p. 1798, 1900.

0.1530 gram of substance gave 0.2343 gram CO_2 and 0.0688 gram H_2O.

	Calculated for $C_{11}H_{15}N_2BrO_4$.	Found.
C	41.39	41.28
H	4.71	4.99

0.2000 gram of substance in 3 cc. pyridine rotated in 0.5 dm. tube with D-light:

After ten minutes	+1.06°
After twenty-four hours	+0.26°
After forty-eight hours	+0.26°

d-Lyxose-*p*-nitrophenylhydrazone melted at 169°C. (corr. 172°).

0.1547 gram of substance gave 0.2655 gram CO_2 and 0.0768 gram H_2O.

	Calculated for $C_{11}H_{15}N_3O_6$.	Found.
C	46.36	46.27
H	5.26	5.51

p-Bromphenylhydrazones of Pentoses.

PENTOSE	M. P. °C.	SOLVENT	CONCENTRATION	$[\alpha]_D$ IN 0.5 DM. TUBE	
				Initial	Final
d-Lyxose	158	Pyridine	1 gram in 10 cc.	+1.41	+0.34
l-Xylose	128–129	Alcohol	1 gram in 10 cc.	+0.06	+0.06
l-Xylose		Pyridine	1 gram in 10 cc.	−1.22	−0.42 (last reading)
Urine pentose	128–129	Alcohol	1 gram in 10 cc.	−1.00	+1.12
Urine pentose		Pyridine	1 gram in 10 cc.	−0.87	+1.06

Cleavage of the Urine Pentose-Parabromphenylhydrazone with Benzaldehyde.

2.20 grams hydrazone dissolved in about 75 cc. of hot water were heated three quarters of an hour on the water bath with 0.8 gram of benzaldehyde. The hydrazone of benzaldehyde begins to separate at once and at the end of the experiment, after having been filtered off, washed and dried, weighed 1.83 grams, which is 97 per cent of the calculated 1.89 grams. In the filtrate were present by calculation 1.035 grams of pentose. The solution was extracted four times with ether and then concentrated in vacuum to 40 cc., placed in a measuring flask and the volume made up to 50 cc.

This solution showed a rotation in 2 dm. tube at 25° with D-light of + 1.38°.

$$[\alpha]_D^{25} = +33.15°.$$[7]

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[7] This value must be regarded as a minimum.

Reduction of Fehling Solution.

I. 2 cc. of solution, containing 0.0412 gram pentose as calculated from the amount of hydrazone, corresponded to 11.3 cc. $\frac{N}{10}$ NH_4CNS (0.0717 gram Cu).

II. 2 cc. of solution gave 11.2 cc. $\frac{N}{10}$ NH_4CNS (0.0710 gram Cu).

Factor = 0.577.

Attempts to Oxidize the Pentose with Bromine.

Two grams of pentose, calculated from reduction with Fehling solution (the reduced copper being estimated by the Volhard method) in 90 cc. of water, were allowed to stand for two days at 25° with an excess of bromine. After this time, 2 cc. were boiled to remove the bromine and found by reduction to correspond to 11 cc. $\frac{N}{10}$ sulphocyanate solution = 0.0697 gram Cu, or using the above-mentioned factor, 0.0398 gram pentose in 2 cc. or 0.179 gram in 90 cc. (85 per cent of the original amount).

A parallel experiment made with 2 grams of xylose under the same conditions showed only 0.04 gram pentose in the whole solution (12 cc. corresponded to 1.5 cc. $\frac{N}{10}$ sulphocyanate solution).

Attempts to oxidize with nitric acid in the usual manner the sugar syrup obtained from the parabromphenylhydrazone were unsuccessful. Nothing could be isolated from the reaction product.

The Relation between the Reducing Power of d-Xylose to Rotation after Precipitation with Basic Lead Acetate.

Four grams of *d*-xylose dissolved in 1 liter of water were precipitated with basic lead acetate and barium hydrate. The precipitate was decomposed with sulphuric acid, the excessive sulphuric acid removed with lead carbonate, the excess of the latter with hydrogen sulphide, and the solution concentrated to about 75 cc.

1 cc. corresponded to 8 cc. of $\frac{N}{10}$ sulphocyanate solution (Volhard).

1 cc. corresponded to 0.0312 gram of xylose; a in 2 dm. tube and D-light = 1.11.

$$[a]_D = -18.16°.$$

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ON SPHINGOMYELIN.

SECOND PAPER.*[1]

BY P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

Sphingomyelin was first discovered by Thudichum[2] and later studied by Rosenheim and Tebb.[3] Thudichum was also the first to have accomplished a hydrolysis of the substance, and to have attempted the identification of its components. As such he described two basic substances, sphingosine and neurine; an acid, sphingostearic acid; an alcohol, sphingol, and phosphoric acid. Thudichum based his conclusions on analytical data. The data and conclusions will be discussed later in connection with the individual components. Rosenheim and Tebb have improved the method of preparation of the substance. Regarding the components of sphingomyelin these authors refer only to the bases and to the alcohol. They speak of the bases as choline and sphingosine but do not substantiate their conclusions by analytical data, or by any other experimental evidence.

The ultimate aim of the present work was to establish the structure of sphingomyelin. As introductory to this an attempt was made to discover the conditions which would make possible the preparation of a pure sphingomyelin, and further, to establish conditions of hydrolysis leading to a satisfactory yield of its principal components.

The aim of the preliminary work in a measure was accomplished. The conditions were found which made possible the preparation of the phosphatide entirely free from galactosides. The conditions

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* Received for publication, June 16, 1914.

[1] This *Journal*, xv, p. 153, 1913.

[2] Thudichum: *On the Chemical Constitution of the Brain*, London, 1884.

[3] Rosenheim and Tebb: *Quart. Journ. of Physiol.*, i, p. 297, 1908; *Journ. of Physiol.*, xli, *Proc. Physiol. Soc., July 9, 1910.*

were also found which permitted a satisfactory hydrolysis, so that every component could be obtained in quantities permitting its purification. Since the present work principally aimed to establish methods of analysis, it was carried out on material of only relative purity. Further purification is accompanied with great loss of material and time, and the presence of a little impurity does not affect much the course of hydrolysis and the yields of the principal components. On the other hand, the results of the analysis of even slightly impure material do not permit of a final conclusion regarding the number and the character of the components of sphingomyelin. In fact, the results of the present hydrolysis raise the question whether or not there exist more than one sphingomyelin, or whether the substance has the structure of a polyphosphatide, of which every unit is composed of a different base and of a different organic acid.

Properties of Sphingomyelin.

The substance was purified until it gave a negative test with orcin and copper, thus showing the absence in it of a carbohydrate. When obtained in that degree of purity the substance is optically inactive. It has the composition: $C = 64$; $H = 11$; $N = 3.40$; $P = 3.60$; inorganic bases = 3 per cent; the proportion of $N : P = 2 : 1$ (approximately). It contains no free amino-nitrogen in the molecule, and contains three methyl groups to two atoms of nitrogen, hence one nitrogen atom in the form of choline. The substance, dissolved in glacial acetic acid and alcohol, absorbs hydrogen.

Results of the Hydrolysis of the Substance.—For hydrolysis a substance was employed that frequently showed a composition not much different from the purest material. The least purified material employed in the work was in its ultimate composition practically identical with the substance of Thudichum. This is seen from the following data:

TABLE I.

	C	H	N	P	O	INORGANIC BASES	METHOD OF PURIFICATION
Thudichum	65.37	11.29	2.96	3.24	17.04		
Present work:							
Sample 1	66.90	11.42	3.50	3.80		3.0	Pyridine and chloroform; negative orcin test.
Sample 2	66.90	11.12	3.37	3.73		4.5	Ligroin and alcohol; very faint orcin and copper test.
Sample 3	66.65	11.58	3.48	3.58		3.5	
Sample 4	67.60	11.71	3.64	3.36		0.0	
Sample 5	67.61	11.59	3.29	3.78		0.0	
Sample 6	67.44	11.76	3.29	3.69		0.0	
Sample 7	67.27	11.00	3.22	3.71		0.0	

On hydrolysis the following acid substances were obtained: Phosphoric acid, and two organic acids, cerebronic and lignoceric. It is noteworthy that the mixture of the two acids as obtained in our experiments had approximately the following composition: $C = 76.5$; $H = 12.6$ per cent. These figures are nearly identical with those obtained by Thudichum, which caused him to regard the mixture as an isomeric stearic acid.

Of the basic substances one was identified without great difficulty —this was choline. On the other hand, it was found very difficult to obtain conclusive evidence regarding the character of the other base or of the other bases. A method of hydrolysis which was successfully employed in preparation of sphingosine from cerebrosides led to a substance with a constant composition. The analytical data of the sulphate and hydrochloride of the substance corresponded to the formula $C_{15}H_{31}NO_2$. However, this view soon was abandoned as erroneous. It was found that the base was contaminated with traces of phosphorus-containing bodies, although the total ash of the substance did not exceed 2 per cent. After much experimenting the conditions were discovered which permitted a complete hydrolysis of the original material and which at the same time left a reasonable proportion of the basic substances intact. However, the material obtained by this process had a varying composition. The base was always analyzed as the sulphate. The carbon of this fluctuated between 62.5 to 63.5 per cent and the hydrogen between 10.3 to about 11.0 per cent. It was found convenient to reduce the bases with hydrogen and palladium before fractionation. Out of a chloroform-alcohol mixture it was possible to separate a sulphate having the following composition: C = 63.60–64; H = 11.5; N = 4.2–4; S = 4.4–4.9. A substance of this composition corresponds to a sulphate of a base having the composition: $(C_{17}H_{37}NO)_2H_2SO_4$.

As yet we were never in possession of the material in a quantity sufficient for a satisfactory diagnosis of it. However, since the methods of preparation of both sphingomyelin, and its basic components are perfected, one may hope in the near future to reach a definite conclusion regarding the nature of the basic substances of sphingomyelin. Again on this occasion it is worthy of note that the basic substance obtained by Thudichum had an ultimate composition nearly identical with that of the fraction of the crude bases obtained in the present work. Thudichum regarded his substance as impure sphingosine. The present work favors rather the view that the fraction represents a mixture of two bases.

CONCLUSIONS.

The results of the present work permit of the following conclusions:

1. Sphingomyelin having the composition of the substance obtained by Thudichum is contaminated with galactosides.
2. It is not certain whether the sphingomyelin fraction of the brain lipoids, after it is freed from galactosides, contains only one phosphatide.
3. Sphingomyelin, apparently containing a very insignificant proportion of galactosides, is composed of the following substances: phosphoric acid, lignoceric and cerebronic acids, choline, sphingosine, and perhaps another base having the composition of $C_{17}H_{35}NO$. It remains to be established whether or not the latter substance is impure sphingosine, and if it is a different base, whether or not it is a primary constituent of sphingomyelin.

EXPERIMENTAL PART.

Preparation of sphingomyelin requires six principal stages:

1. Desiccated brain tissue is exhaustively extracted with boiling

alcohol. Each extraction lasted about thirty minutes. On cooling this extract forms a precipitate.

2. The precipitate is exhaustively extracted with ether and acetone.

3. The residue is dissolved in hot technical pyridine and allowed to cool. On standing there forms a precipitate.

4. This precipitate is dissolved in hot glacial acetic acid and allowed to cool. There forms a precipitate. The mother liquor contains sphingomyelin. It is concentrated under diminished pressure and transferred into acetone. A precipitate forms consisting of crude sphingomyelin.

5. The substance obtained through treatment (4) is dissolved in 5 parts of ligroin and 1 of alcohol. An excess of 98 per cent alcohol is then added as long as a precipitate is formed. The mother liquor from the precipitate contains purified sphingomyelin. It is concentrated under diminished pressure and transferred into acetone. The purity of the material obtained in these stages depends largely on the nature of ligroin employed for solution. However, the poorest material obtained in this phase had the composition of Thudichum's sphingomyelin.

6. The final purification is accomplished by recrystallization of the substance from a solution containing equal parts of pyridine (Kahlbaum's) and chloroform.

Composition of Sphingomyelin.—Table I contains the results of analysis of a great number of samples of sphingomyelin. For economy of space only the final values are given. Sample 1 represents sphingomyelin which gave a negative orcin test. All the other samples gave a positive test, though of varying intensity. In some the color test was very faint.

The carbon and hydrogen estimations were made by the Dennstedt method; nitrogen by the Kjeldahl process, and phosphorus by the fusion method.

The *methyl estimation* was carried out according to the directions of Herzig and Meyer. The only modification in the process consisted in the use of two individual Rose metal baths for heating the bulbs, instead of the sand bath employed originally. The temperature of a sand bath differs too much in different layers to permit of reliable information regarding the temperature of that part of the apparatus where the reaction takes place. The purity of reagents was tested.

TABLE II.

NUMBER OF SAMPLE	WEIGHT OF SUBSTANCE	WEIGHT OF AGI	PER CENT NITROGEN	CH_3	
				Calculated	Found
	grams	*grams*		*per cent*	*per cent*
4	0.2724	0.1954	3.36	5.40	4.57
5	0.2200	0.1638	3.29	5.27	4.75
6	0.3375	0.2456	3.29	5.27	4.64
7	0.2706	0.2542	3.32	5.33	5.99

Isolation and Identification of Choline.—35.0 grams of sphingomyelin were taken up with an equal weight of recrystallized barium hydrate in 200 cc. of water and heated in an autoclave at 120° for six hours. The reaction product was filtered. The filtrate was freed from barium and concentrated. The residue was extracted with absolute alcohol, the alcoholic extract filtered and concentrated. The residue was extracted with alcohol, again concentrated, and the final residue acidulated with hydrochloric acid and treated with chloroplatinic acid. The precipitate was recrystallized out of 60 per cent alcohol.

0.1196 gram of substance gave 0.0494 gram H_2O, 0.0846 gram CO_2 and 0.0382 gram Pt.

	Calculated for $(C_5H_{14}NOCl)_2PtCl_4$.	Found.
C	19.48	19.29
H	4.58	4.13
Pt	31.65	32.00

Hydrolysis of Sphingomyelin.—Only the process as finally adopted will be given here. Many other conditions have been tested out and employed in course of the work, but all were found less satisfactory for one reason or another.

A given weight of the substance, an equal weight of barium hydrate crystals and ten times its weight of water were placed in an autoclave provided with a stirring arrangement, and heated for forty-eight hours at 120°C. The reaction product was filtered. The filtrate contained choline, the residue the acids, the bases, and some intermediate products of hydrolysis. The entire residue was then taken up in a solution of 4 parts of 10 per cent hydrochloric acid and 1 part of 95 per cent alcohol, transferred to a hydrolysis flask provided with return condenser and mechanical stirrer and heated over an open flame for forty-eight hours. At the end of that time a layer of oil formed on the surface of the liquid. On cooling it formed a solid cake. It was found that the cake consisted of fatty acids, some esters, and the bases that were not choline. In order to saponify the esters the cake was dissolved in methyl alcohol, an excess of barium hydrate in methyl alcohol added, and the mixture heated (in a hydrolysis flask provided with a return condenser) for six hours in a water bath.

Separation of the Acids from the Bases and their Purification.—The acids are obtained by treating the reaction mixture of the previous experiment with acetone. A precipitate is formed which consists principally of fatty acids. For purification the barium salts are decomposed with hydrochloric acid and the fatty acids extracted with ether. The mixed acids obtained in this manner have as a rule the following composition: $C = 76.2–76.8$; $H = 12.6–12.8$. The isolation of lignoceric acid is accomplished in the following way. The mixed acids are extracted with petrolic ether boiling below 40°C. The extract consists principally of lignoceric acid. In order to purify the acid, the ethereal extract is freed from ether and the residue esterified by boiling with ethyl alcohol to which sulphuric acid was added to make a 5 per cent solution. On cooling to about 15° the ester separates in bright scales. These are recrystallized once out of slightly acidulated alcohol and once out of acetone. The substance melts at 56° and has the following composition:

0.1170 gram of substance gave 0.1342 gram H_2O and 0.3372 gram CO_2 (Dennstedt).

	Calculated for $C_{24}H_{47}O_2.C_2H_5$.	Found.
C....................................	78.79	78.59
H....................................	13.13	12.83

The cerebronic acid was separated from another experiment. The product obtained after hydrolysis with 4 parts of dilute hydrochloric acid and 1 part of alcohol was dissolved in methyl alcohol. This solution contained the bases, and the fatty acids and their esters. The free acid is (largely) cerebronic acid. Lignoceric acid is present principally in form of its ester. Hence adding to a methyl alcoholic solution of the products of hydrolysis a solution of barium hydrate in methyl alcohol one obtains the barium salt of cerebronic acid. The adhering lignoceric acid ethyl ester is then removed by means of ether. The barium salt of cerebronic acid is then decomposed by means of hydrochloric acid and the acid extracted with ether. If the resulting product still contains an admixture of lignoceric acid the latter can be removed as its lithium salt. In the experiment here reported this was not necessary. The acid obtained in this manner had the composition of cerebronic acid.

0.1088 gram of substance gave on combustion (Dennstedt) 0.1210 gram H_2O and 0.3022 gram CO_2.

	Calculated for $C_{25}H_{50}O_3$.	Found.
C..	75.33	75.72
H..	12.50	12.44

For the molecular weight estimation the substance was converted into its lead salt, which was washed first with water and then with acetone. The lead salt was finally decomposed in the usual way and the resulting acid employed for molecular weight estimation.

1.0266 grams of substance were dissolved in benzene and methyl alcohol and titrated first with $\frac{N}{2}$ and towards the end with $\frac{N}{10}$ alkali. It consumed the equivalent of 25.5 cc. of $\frac{N}{10}$ alkali.

	Calculated for $C_{25}H_{50}O_3$.	Found.
Mol. Wt..	398	402

The Separation of the Bases.—A great many experiments were made with a view of obtaining the principal base in a pure condition. Only the final form of analysis will be given here. The crude bases are obtained in the following way. The filtrate obtained after filtering off the barium salts of the fatty acids is evaporated to dryness under diminished pressure. The residue is redissolved in acetone. The soaps that were not removed by the first acetone treatment remain insoluble on the second extraction. The insoluble part is removed by filtration, and the filtrate is concentrated to dryness. If necessary the acetone treatment may be repeated. The final residue containing the bases and some esters of the fatty acids is dissolved in very little alcohol. From this solution the bases can be isolated in form of their sulphates by adding an alcoholic solution of sulphuric acid until the reaction of the mixture turns acid to litmus. The sulphates obtained in this manner contained about 63 per cent of C and about 10.5 per cent of H. Hence it was concluded to saturate the alcoholic solution of the bases with hydrogen in the presence of palladium. It was found that the fractionation was accomplished easier on the dihydro bases.

The solution of the saturated bases was then treated with alcoholic sulphuric acid and the sulphates of the bases freed from adhering esters by means of dry ether. These mixed sulphates were repeatedly fractionated out of a solution containing equal parts of alcohol and of chloroform. By this treatment on several occasions it was possible to obtain a fraction which in its composition approached that of $(C_{17}H_{37}NO)_2H_2SO_4$ and another fraction which contained only about 62 per cent of carbon and sometimes fractions that analyzed well for sphingosine sulphate.

However, the many purifications consumed so much material that as yet we are not in possession of a quantity sufficient to make derivatives of the bases. It is hoped that this end will be achieved in the future.

Results of Analysis of Several of the Higher Fractions.

Sample No. 565.—0.1174 gram of substance gave on combustion 0.1213 gram H_2O and 0.2770 gram CO_2.

0.1000 gram of substance employed for Kjeldahl nitrogen estimation, required 3 cc. $\frac{N}{10}$ H_2SO_4 for neutralization.

Sample No. 657.—0.1182 gram of substance gave 0.1200 gram H_2O and 0.2781 gram CO_2.

0.1495 gram of substance used for Kjeldahl nitrogen estimation, required for neutralization 4.95 cc. $\frac{N}{10}$ H_2SO_4.

Sample No. 768.—0.1157 gram of substance gave 0.1202 gram H_2O and 0.2690 gram CO_2.

0.1976 gram of substance used for Kjeldahl nitrogen estimation, required 5.85 cc. $\frac{N}{10}$ H_2O_4 for neutralization.

0.2964 gram of substance gave 0.1066 gram $BaSO_4$.

	Calculated for $(C_{17}H_{37}NO)_2 H_2SO_4$.		Found.	
		No. 565.	No. 657.	No. 768.
C	63.80	64.34	64.17	63.40
H	11.85	11.56	11.38	11.63
N	4.37	4.20	4.65	4.15
S	5.00			4.94

The sample No. 768 melted with effervescence at 280° (corr.) and had the following rotation:

0.1000 gram of substance in 2.0 cc. of alcohol containing sulphuric acid (total weight, 1.8305) rotated in 1 dm. tube — 0.70 at t = 25°C.

$$[\alpha]_D^{25} = -12.8°.$$

The number of substances that gave lower values for carbon and hydrogen was very great. There were analyzed at least about thirty samples. However, little significance can be attached to these values, hence they are not reported here.

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PURIFICATION AND MELTING POINTS OF SATURATED ALIPHATIC ACIDS.*

BY P. A. LEVENE AND C. J. WEST.

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In course of our work on lipoids we frequently were in need to refer to the melting points of the known higher fatty acids. On several occasions we had sufficient ground to doubt the correctness of the accepted melting points, and hence we were led to reinvestigate the aliphatic acids beginning with $C_{11}H_{22}O_2$ and ending with $C_{16}H_{32}O_2$ in regard to their physical constants.

The melting points found by us were generally higher than those given in the literature. It was also found that the purest acids after distillation even under very low pressure as a rule do not show their maximum melting points. In order to obtain the maximum several recrystallizations out of acetone were required. An irregularity was observed in connection with the melting point of lauric acid. This acid is considered to have the structure of the normal dodecylic acid. As a rule the melting point of the normal acids with an even number of carbon atoms in the chain is higher than that of the following acid with an uneven number of carbon atoms. Thus, $C_{14}H_{28}O_2 =$ M.P., 58°C, and $C_{15}H_{30}O_2 =$ M.P., 54°C, whereas the melting point of lauric acid, $C_{12}H_{24}O_2$ is 48°C, and that of tridecylic, $C_{13}H_{26}O_2$ is 51°C. We plan to investigate into the causes of this abnormality.

EXPERIMENTAL PART.

The fatty acids used in the following work were prepared by the method recently described by us[1] or were purchased from Kahlbaum or Schuchardt. In each case at least three different lots of the acid

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* Received for publication, June 16, 1914.

[1] Levene and West: this *Journal*, xvi, p. 475, 1914.

were purified using the following methods: (1) The acid was recrystallized from acetone two or three times, distilled in vacuum, and then recrystallized from acetone. (2) The acid was purified through the lead salt (the lead salt in most cases being extracted with acetone to remove any ester present), and then recrystallized several times from dry acetone. (3) The crude acid was distilled in vacuum (Geryke pump) and then recrystallized from acetone until the melting point was constant. We have purposely avoided the use of alcohol as there is always more or less esterification of the acid, even if the acid is boiled with the alcohol for a very short time.[2] The presence of a very small amount of ester naturally would lower the melting point of the acid. It has been our experience that the melting points of the acids are low after the distillation in vacuum. While Holland recommends the distillation of the acid or its ester as a method of purification and says that crystallization is a finishing rather than an initial process of purification, we have found that crystallization is a very important process in cases where the correct melting point is desired. It is true that, in the cases of the lower members of the series, the process is rather wasteful of material, though, of course, all the crude material can be recovered from the mother liquor.

Undecylic Acid, $C_{11}H_{22}O_2$.—Undecylic acid was obtained by reducing undecylenic acid with hydrogen and colloidal palladium. Six cc. of Schuchardt's acid were dissolved in about 25 cc. absolute alcohol, the solution warmed to 60–70° and shaken with 0.1 gram colloidal palladium, dissolved in a little water, in an atmosphere of hydrogen. When no more hydrogen was absorbed, the reaction product was filtered from the palladium (a few drops of acetic acid may be necessary to coagulate the palladium), warmed on the water bath and treated with a slight excess of methyl alcoholic lead acetate. The lead salt is decomposed as usual with hydrogen sulphide in toluene. The acid boiled at 164° under 15 mm. pressure and melted at 28–29°. Recrystallized from dry acetone at —10° it melts at 29–30°. Because of its great solubility it is not practical to purify it further. Krafft[3] gives 28.5 as the melting point.

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[2] Holland: *Journ. of Ind. and Eng. Chem.,* iii, pp. 171–3, 1911. This does not hold true for the higher fatty acids (above C_{20}).

[3] *Ber. d. deutsch. chem. Gesellsch.,* xi, p. 2219, 1878; xii, p. 1668, 1879.

0.1200 gram of substance gave 0.3115 gram CO_2 and 0.1262 gram H_2O.

0.9800 gram of the acid, dissolved in absolute methyl alcohol and benzene, required 53 cc. $\frac{N}{10}$ NaOH, using phenolphthalein as an indicator.

	Calculated for $C_{11}H_{22}O_2$.	Found.
C	70.9	70.80
H	11.8	11.77
Mol. Wt.	186.0	185.00

The acid was characterized by changing it into the *amide*,[4] which, recrystallized from absolute alcohol, melted at 103°.

This method of reducing unsaturated fatty acids has also been successfully used in the preparation of behenic acid from erucic acid. It is as convenient as the palladium black method, which is recommended by Parnas,[5] or the use of finely divided nickel heated to 170°, as used by Pickard and Kenyon.[6]

Lauric Acid, $C_{12}H_{24}O_2$.—Kahlbaum's acid melts at 46–48°, and boils at 141–142° under a pressure of 0.6–0.7 mm. Twice recrystallized out of acetone it melts at 47.5–48°. The other methods of purification gave a similar melting point. Krafft[7] gives a melting point of 43.5°.

Tridecylic Acid, $C_{13}H_{26}O_2$.—Tridecylic acid was prepared by the oxidation of α-hydroxy-myristic acid with potassium permanganate in acetone solution. In this case the potassium salt of the acid is soluble in acetone and most of the desired product is found in the filtrate from the manganese dioxide. The acid boils at 202–203° under 17 mm. pressure, and melts at 47–48°. Twice recrystallized from acetone it melts at 50–51°. The melting points given in the literature are, Krafft,[8] 40.5°; Le Sueur,[9] 42.5°.

0.557 gram of substance, dissolved in a mixture of equal parts of benzene and methyl alcohol, required 26 cc. $\frac{N}{10}$ NaOH for neutralization.

	Calculated for $C_{13}H_{26}O_2$.	Found.
Mol. Wt.	214.0	214.3

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[4] Ehestädt: Dissertation, Freiburg, 1886.

[5] *Biochem. Zeitschr.*, xxii, p. 428, 1909.

[6] *Journ. Chem. Soc.*, ciii, p. 1947, 1913.

[7] *Ber. d. deutsch. chem. Gesellsch.*, xii, p. 1666, 1879; xiii, p. 1415, 1880; Heintz: *Ann. d. Chem.*, xcii, p. 294, 1854.

[8] *Ber. d. deutsch. chem. Gesellsch.*, xii, p. 1670, 1879.

[9] *Journ. Chem. Soc.*, lxxxvii, p. 1898, 1905.

The acid was characterized as the amide, which, recrystallized from absolute alcohol, melts at 98.5°. E. Lutz[10] gives the same melting point for the product obtained from myristic acid amide.

Myristic Acid, $C_{14}H_{28}O_2$.—The sample of Schuchardt's acid used melted at 53.5–54° and boiled at 185–187° at 0.8–0.9 mm. pressure. The distilled product melted at 56–57°. This melting point was raised to 57.5–58° after three recrystallizations. The melting point given in the literature is 53.5–54°.[11]

Pentadecylic Acid, $C_{15}H_{30}O_2$.—Pentadecylic acid was obtained by the oxidation of α-hydroxy-palmitic acid.

Methyl α-hydroxy-palmitate, prepared in the usual way by boiling the acid with absolute methyl alcohol containing about 10 per cent sulphuric acid, forms colorless crystals from absolute methyl alcohol, then from acetone, melting at 59–60°.

0.1200 gram of substance gave 0.3128 gram CO_2 and 0.1276 gram H_2O.

	Calculated for $C_{17}H_{34}O_3$.	Found.
C	71.25	71.09
H	11.97	11.90

Ethyl α-hydroxy-palmitate was prepared as given above. Recrystallized from absolute alcohol it melts at 55.5–56.5°.

0.1200 gram of substance gave 0.3161 gram CO_2 and 0.1287 gram H_2O.

	Calculated for $C_1H_{36}O_3$.	Found.
C	71.93	71.84
H	12.08	12.00

Oxidized in the usual way, and purified by distillation and crystallization from acetone, a product was obtained which melted at 53°. This was fractionally precipitated with lead acetate and the three products obtained fractionally crystallized from dry acetone. No change in the melting point was observed.

The acid was then prepared according to the directions given for oxidation of sphingosine:[12] 10 grams of the hydroxy acid were dissolved in 100 cc. of glacial acetic acid and a solution of 5.5 grams of chromic acid in acetic acid slowly added from a dropping funnel, the acetic acid removed with steam and the chromium containing product distilled in vacuum. This acid, when redistilled and twice recrystallized from acetone, again melted at 53–54°.

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[10] *Ber. d. deutsch. chem. Gesellsch.*, xix, p. 1439, 1886.

[11] Krafft: *Ber. d. deutsch, chem. Gesellsch.*, xii, p. 1669, 1879; xiii, p. 1415, 1880; Noerdlinger: Ibid., xix, p. 1893, 1886; Heintz: *Ann. d. Chem.*, xcii, p. 292, 1854.

[12] This *Journal*, xvi, p. 549, 1914.

0.1200 gram of substance gave 0.3284 gram CO_2 and 0.1319 gram H_2O.
1.000 gram of the acid required 41.3 cc. $\frac{N}{10}$ NaOH for neutralization.

	Calculated for $C_{15}H_{30}O_2$.	Found.
C	74.4	74.3
H	12.4	12.3
Mol. Wt.	242.0	242.1

The melting points given for pentadecylic acid are: Krafft,[13] 51°; Le Sueur,[14] who prepared it by oxidizing the aldehyde, 53°; and Majima and Nakamura,[15] who prepared it by hydrolysis of the cyanide, 52°.

The amide obtained from this acid melted at 102–103°, which is that given in the literature.

Palmitic Acid, $C_{16}H_{32}O_2$.—Palmitic acid, as above, showed a melting point of 63.5–64°.

The following table gives a comparison of our melting points with those previously recorded in the literature:

ACID	MEYER-JACOBSON	LE SUEUR	LEVENE AND WEST
$C_{11}H_{22}O_2$	28.0°		29.3°
$C_{12}H_{24}O_2$	44.0°		48.0°
$C_{13}H_{26}O_2$	40.5°	42.5°	51.0°
$C_{14}H_{28}O_2$	54.0°	53.5–54°	58.0°
$C_{15}H_{30}O_2$	51.0°	53.0°	54.0°
$C_{16}H_{32}O_2$	62.6°	62.5°	63–64°

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[13] *Ber. d. deutsch. chem. Gesellsch.,* xii, p. 1671, 1879.

[14] *Journ. Chem. Soc.,* ciii, p. 1898, 1905.

[15] *Ber. d. deutsch. chem. Gesellsch.,* xlvi, p. 4089, 1913.

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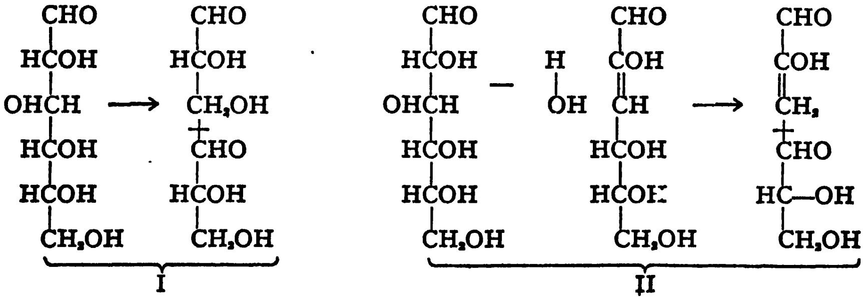
ON THE ACTION OF TISSUES ON METHYL GLUCOSIDES, TETRAMETHYL GLUCOSE, AND NATURAL DISACCHARIDES.*

BY P. A. LEVENE AND G. M. MEYER.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The volume of work done on the mechanism of sugar combustion in the animal organism is very considerable. The information thus far furnished by it is insignificant. It is known that in the process of sugar oxidation lactic acid formation does take place, probably through the intermediate formation of methyl glyoxal. The steps that lead up to methyl glyoxal are not known. The glucose molecule may be dissociated into two molecules of glyceric aldehyde. It may also undergo such a transformation as will lead to its further dissociation into methyl glyoxal and into glyceric aldehyde. The glucose molecule may also suffer a change leading to its dissociation into two molecules of methyl glyoxal. The three possibilities may be graphically expressed in the following manner:



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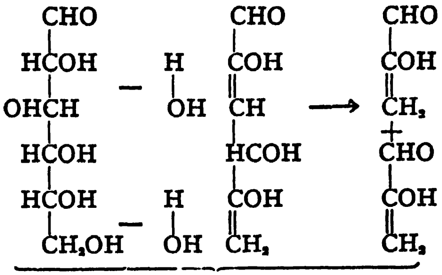
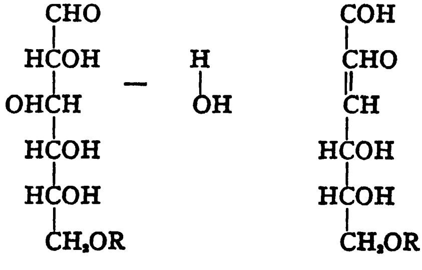
* Received for publication, June 16, 1914.



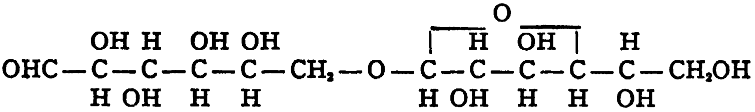
III

A priori all these reactions are possible. In reality there is no experimental evidence either for or against any one of the possibilities. From the graphic expressions of the reactions it is made obvious that the hypothetic intermediate body is formed through a loss of one or more molecules of water by the original hexose molecule. This reaction presumes a certain degree of mobility of the various atoms and hydroxyl groups in the sugar molecule. On the basis of this one conceives the activity of sugar-splitting enzymes as playing on the mobility of the various hydrogen atoms or hydroxyl radicles of the sugar molecule. Naturally one should expect that by stabilization of one or more hydroxyl groups the resistance of the hexose molecules against the action of the enzyme will be enhanced. For instance, one may conceive that by stabilizing the hydroxyl in ϵ-position the first hydrogen and hydroxyls in position α and β will function normally and hence the altered molecule may still yield half of the normal quantity of methyl glyoxal and of lactic acid. This is made obvious by the following graphic expression:



These considerations led us to undertake the study of the action of tissue extracts on sugar derivatives in which one or more hydroxyl groups are stabilized. There were used in course of the experiments α- and β-methyl-*d*-glucosides, tetramethyl-*d*-glucose, natural disaccharides: sucrose, lactose, and maltose, and finally *d*-glucose-phosphoric acid (according to Neuberg[1]). It was also planned to prepare partially methylated glucose according to the process of Irvine.[2] However, the results of the experiments on the enumerated substances were so uniform and so conclusive that further experimentation in this direction seemed unnecessary.

It was found that β-methyl-*d*-glucose and maltose show some loss of the glucose contained in them. All other sugar derivatives and disaccharides remained unchanged. The behavior of lactose in this respect is most interesting, for in this disaccharide the glucose molecule is practically unaltered with the exception of the alcoholic group in position δ or ε, which is in glucosidic union with galactose. And yet this union is sufficient to protect lactose against the action of glucose-cleaving enzymes:



$$\mathrm{OHC-\overset{OH}{\underset{H}{C}}-\overset{H}{\underset{OH}{C}}-\overset{OH}{\underset{H}{C}}-\overset{OH}{\underset{H}{C}}-CH_2-O-\overbrace{\underset{H}{C}-\overset{H}{\underset{OH}{C}}-\overset{OH}{\underset{H}{C}}-\underset{H}{C}}^{O}-\overset{H}{\underset{OH}{C}}-CH_2OH}$$

Glucose rest. Galactose rest.

The reason for the exceptional behavior of β-methyl glucoside and of maltose is undoubtedly to be found in the fact that both these glucosides are cleaved by tissue enzymes into their components.

Thus it is evident from our experiments that only free glucose is converted into lactic acid; glucose in ester and ether linking with one or more groups is not affected by tissue enzymes. Our experiments are in harmony with the experiments of F. Voit.[3] This author found that disaccharides when injected intravenously into a dog reappear in a large proportion unchanged in the urine. On the other hand, maltose in a considerable proportion was utilized by the organism. Undoubtedly the exceptional position of maltose in the experiments of Voit is due to the same factor as in our experiments.

The results of our experiments seem to be of special interest at the present moment when several authors have advanced the view that glucose prior to its combustion by the animal organism enters into some rather mysterious chemical union with other substances. It is hard to reconcile the results of our experiments with such speculations.

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[1] Neuberg: *Biochem. Zeitschr.*, xxvi, p. 523, 1910.

[2] Purdie and Irvine: *Jour. chem. Soc.*, lxxxiii, p. 1028.

[3] Voit, F.: *Deutsch. Arch. f. klin. Med.*, lviii, p. 522, 1897.

EXPERIMENTAL PART.

Tissues.—Rabbit kidneys were removed aseptically from exsanguinated animals, finely cut and immediately added to the sugar solutions.

Sugar Solutions.—All disaccharide solutions were of 5 per cent in 10 per cent Henderson phosphate solution and sterilized by filtering through sterile Berkefeld filters. Their sterility was tested before adding the tissues by means of cultures and smears. The tetramethyl glucose was prepared according to the method of Purdie and Irvine. It was redistilled under diminished pressure and boiled constant at 122° at 0.5 mm., and melted at 81°. Glucosephosphoric acid was prepared according to Neuberg. A PO_4 determination of the calcium salt showed that it contained 8.9 per cent P. Five and eight per cent solutions were used. For the other sugars, preparations of Kahlbaum were used. All solutions were allowed to stand at 37° for thirty-six hours. Samples of each were removed for analyses immediately after adding the kidney tissue. In the experiments with tetramethyl glucose and glucosephosphoric acid the sugar solution was divided into equal parts, and to each was added one kidney. One portion was reserved as control and analyzed immediately. This procedure was followed to eliminate any possible contamination of the main portion while removing the control sample for analysis.

Methods of Analysis.—The reducing power of the sugar solutions was determined by Fehling solution and estimating the reduced copper according to Volhard. Determination of reducing power was also made on the sugar after hydrolysis with 2 per cent hydrochloric acid according to the standard methods for each particular sugar.

Bacteriological Control.—All solutions were tested after thirty-six hours' incubation by means of cultures and only those which were found sterile received further consideration.

The bacteriological examinations were made by Dr. Martha Wollstein to whom we desire to express our appreciation.

Maltose.

	CC. USED	$\frac{N}{10}$ NH_4CNS	$\frac{N}{10}$ NH_4CNS PER CC.	DIFFERENCE
I. Before	2	17.30	8.65	
After	2	21.80	10.90	+2.25
Hydrolyzed:				
Before	1	14.90	14.90	
After	1	13.00	13.00	−1.90
II. Before	2	14.00	7.00	
After	2	17.00	8.50	+1.50
Hydrolyzed:				
Before	1	14.50	14.50	
After	1	12.70	12.70	−1.80
III. Before	2	14.00	7.00	
After	2	16.80	8.40	+1.40
Hydrolyzed:				
Before	1	14.50	14.50	
After	1	11.50	11.50	−3.00

Lactose.

I. Before	2	18.00	9.00	
After	2	18.00	9.00	
Hydrolyzed:				
Before	1	12.30	12.30	
After	1	12.20	12.20	
II. Before	2	17.80	8.90	
After	2	17.70	8.85	
Hydrolyzed:				
Before	2	26.50	26.50	
After	2	26.70	26.50	
III. Before	2	18.40	9.20	
After	2	18.40	9.20	
Hydrolyzed:				
Before	1	14.00	14.00	
After	1	13.80	13.80	

Sucrose.

I. Before	4	0.00	0.00	
After	4	0.00	0.00	
Hydrolyzed:				
Before	1	14.40	14.40	
After	1	14.40	14.40	
II. Before	4	0.00	0.00	
After	4	0.00	0.00	
Hydrolyzed:				
Before	1	14.80	14.80	
After	1	14.60	14.60	

β-Methyl Glucoside.

	CC. USED	$\frac{N}{10}$ NH_4CNS	$\frac{N}{10}$ NH_4CNS PER CC.	DIFFERENCE
I. Before	2	0.00	0.00	
After	2	1.50	0.75	+0.75
Hydrolyzed:				
Before	1	13.30	13.30	
After	1	10.30	10.30	−3.00
II. Before	5	0.00	0.00	
After	5	1.20	0.24	+0.24
Hydrolyzed:				
Before	1	13.90	13.90	
After	1	13.00	13.00	−0.90

α-Methyl Glucose.

I. Before	5	0.00	0.00	
After	5	0.00	0.00	
Hydrolyzed:				
Before	1	10.90	10.90	
After	1	10.70	10.70	
II. Before	5	0.00	0.00	
After	5	0.00	0.00	
Hydrolyzed:				
Before	1	11.40	11.40	
After	1	11.60	11.60	

Tetramethyl Glucose.

I. Before	5	14.00	2.80	
After	5	14.20	2.84	
II. Before	4	14.60	3.65	
After	4	14.80	3.70	
III. Before	5	8.60	1.72	
After	5	8.90	1.78	

Glucose-Phosphoric Acid.

I. Before	2	8.50	4.25	
After	2	8.30	4.15	

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, August, 1914, Vol. xviii, No. 3, pp. 477-480.]

ON CEREBRONIC ACID.

FOURTH PAPER.[1]

ON THE CONSTITUTION OF LIGNOCERIC ACID.*

BY P. A. LEVENE AND C. J. WEST.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In a previous publication[2] the conclusion was reached that lignoceric acid had the structure of the normal tetracosanic acid. The view was based on the melting point of the tetracosane obtained on reduction of lignoceric acid. The melting point of the hydrocarbon obtained from lignoceric acid on reduction with hydroiodic acid was 51°C and that given in the literature for the normal tetracosane was also 51°C. Hence the two substances were considered identical. Since then Hans Meyer[3] has published the results of his work on lignoceric acid which led him to the belief that lignoceric acid was not identical but isomeric with the normal tetracosanic acid. This conclusion did not harmonize with the one based on the melting point of the hydrocarbon.

Hence there arose the suspicion that the melting point found by previous observers for the normal tetracosane was incorrect. This consideration led us to undertake a revision of the melting points of a number of the higher hydrocarbons. The work is carried out in coöperation with Mr. J. Van der Scheer. In this communication we report only the results obtained on the hydrocarbon obtained through the action of magnesium on dodecyl iodide and on the hydrocarbon obtained from lignoceric acid. This latter hydro-

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* Received for publication, June 16, 1914.

[1] The preceding papers are to be found in this *Journal*, xii, p. 381, 1912; xiv, p. 257, 1913; xv, p. 193, 1913. In the table in paper III, the melting points of the two esters are interchanged.

[2] This *Journal*, xv, p. 193, 1913.

[3] H. Meyer, L. Brod, and W. Soyka: *Monatsh. f. Chem.*, xxxiv, p. 1113, 1913.

carbon was obtained by reducing lignoceric ester to the corresponding alcohol; the alcohol was then converted into the iodide, and this reduced to the hydrocarbon.

It was found that the hydrocarbon prepared in this manner had the same melting point as found by us previously, namely, 51°C. On the other hand, the melting point of the normal hydrocarbon was found at 55°C. On the basis of this, our original view regarding the structure of lignoceric acid has to be abandoned, and lignoceric acid will have to be regarded as isomeric and not identical with the normal tetracosanic acid. Furthermore, the original view on the structure of cerebronic acid will have to be modified, as in this acid also the carbon atoms are united in a branched and not in a normal chain.

EXPERIMENTAL PART.

Dodecyl Iodide.—Ten grams of dodecyl alcohol, 1.4 grams of red phosphorus and 7 grams of iodine were heated in a metal bath at 170° for one hour. The reaction product was taken up in ether, the solution washed with water, the iodine removed with sodium thiosulphate and the solution dried with anhydrous sodium sulphate. After removing the ether, the iodide boiled at 145–150° under 0.7 mm. pressure.

I. 0.1790 gram of substance gave 0.1418 gram AgI (Carius).
II. 0.1820 gram of substance gave 0.1432 gram AgI (Carius).

	Calculated for	**Found.**	
	$C_{12}H_{25}I$.	**I.**	**II**
I	42.79	42.82	42.53

A preparation in which 10 grams of the alcohol, 40 cc. glacial acetic acid and 120 grams of hydroiodic acid (d. 1.7) were boiled for six hours, contained only 37.70 per cent iodine, showing that part of the iodide had been reduced to the corresponding hydrocarbon. It was not possible to separate these by fractional distillation.

Normal Tetracosane.—This hydrocarbon was prepared by the action of magnesium upon the above iodide in ordinary dry ether.[4]

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[4] This is a general reaction for aliphatic iodides above hexyl iodide, cf. Wren, *Organometallic Compounds of Zinc and Magnesium*, p. 14.

The ether need not be as carefully dried as for the usual Grignard reaction. 0.9 gram of magnesium and about 100 cc. ether are placed in a double-necked flask, fitted with a condenser and a stirrer, and 9 grams of dodecyl iodide, dissolved in ether, gradually added. The reaction is warmed on the water bath until all the magnesium has gone into solution. The reaction product was allowed to stand over night. On cooling there appeared in the ether a deposit of brilliantly shining scales (tetracosane). The mixture was then transferred into acidulated water, and washed in a separatory funnel until all magnesium was removed. By this treatment the Grignard magnesium complex is decomposed. The ethereal solution was warmed and dried with anhydrous sodium sulphate, and then allowed to stand over night in the refrigerator. Tetracosane appeared in the form of bright shining scales. All the dodecane remained in solution. Tetracosane was then purified by fractional distillation. It boiled at 237–240°C under 15 mm. pressure. It was again recrystallized out of ether. The substance melted at 55°C. The melting point given in literature is 51.1°C.[5]

0.1123 gram of substance gave 0.3501 gram CO_2 and 0.1484 gram H_2O.

	Calculated for $C_{24}H_{50}$.	Found.
C	85.10	85.02
H	14.90	14.79

Iso-Tetracosyl Alcohol.—Thirty grams of ethyl lignocerate were reduced with 30 grams sodium and amyl alcohol according to Bouveault and Blanc.[6] The alcohol was extracted from the soap with ether, the solution dried and after the removal of the ether, distilled in vacuum. It boils at 220° under 0.8 mm. pressure. Recrystallized from ligroïn or chloroform, it melts at 72°. The yield is about 4 grams. Since nearly all the acid is recovered, the method is not so wasteful of material as the above yield would indicate.

0.1200 gram of substance gave 0.3571 gram CO_2 and 0.1218 gram H_2O.

	Calculated for $C_{24}H_{49}OH$.	Found.
C	81.26	81.16
H	14.22	14.30

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[5] Krafft: *Ber. d. deutsch. chem. Gesellsch.*, xv, p. 1720, 1882; Mabery: *Amer. Chem. Journ.*, xxviii, p. 165, 1902.

[6] *Compt. rend. Acad. d. sci.*, cxxxvi, p. 1676, 1903; cxxxvii, pp. 60 and 328, 1903.

Iso-Tetracosyl Iodide.—This was prepared as above, using 9 grams of the alcohol, 3.5 grams of iodine and 1 gram of red phosphorus. Recrystallized from ether, the iodide melts at 48°.

0.1704 gram of substance gave 0.0870 gram AgI (Carius).

	Calculated for $C_{24}H_{49}I$.	Found.
I	27.34	27.60

Iso-Tetracosane.—The hydrocarbon corresponding to lignoceric acid has already been described by us as melting at 51°. At that time it was prepared by the action of concentrated hydroiodic acid upon the acid. We have now prepared it by the action of zinc upon the iodide in glacial acetic acid containing hydrochloric acid. The iodide and zinc are suspended in the acetic acid, and hydrochloric acid gas passed in so that there is a constant evolution of hydrogen. At the end of the reduction, the acid is distilled off in vacuum, the hydrocarbon taken up in ether and washed with water. The hydrocarbon was purified by distillation at 9 mm. pressure and 222–225°C. The distillate was recrystallized out of ether. Isotetracosane separated out in shining plates and has a melting point of 51–51.5°C.

0.1133 gram of substance gave 0.3525 gram CO_2 and 0.1488 gram H_2O.

	Calculated for $C_{24}H_{50}$.	Found.
C	85.10	84.84
H	14.90	14.70

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, August, 1914, Vol. xviii, No. 3, pp. 481–484.]

ON SPHINGOSINE.

THIRD PAPER.[1]

THE OXIDATION OF SPHINGOSINE AND DIHYDROSPHINGOSINE.*

BY P. A. LEVENE AND C. J. WEST.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

In the previous work on the oxidation of sphingosine and dihydrosphingosine evidence was furnished that tridecylic acid was formed from the first substance and under the same conditions pentadecylic acid was obtained from the second. On the basis of this the structure of sphingosine was expressed in the following manner:

$$C_{12}H_{25}CH = CH.CHOH.CHOH.CH_2NH_2.$$

The character of the carbon chain and the configuration of the substituting groups was not established. The failure to determine the nature of the carbon chain was due to the fact that the melting point found by us for the tridecylic acid obtained on oxidation of sphingosine was higher than the one at that time accepted for the normal tridecylic acid and the same applied to the pentadecylic acid obtained on oxidation of dihydrosphingosine.

Hence we were led to revise the older data on the melting points of the aliphatic acids from $C_{11}H_{22}O_2$ to $C_{18}H_{36}O_2$. The results are reported in a separate communication. On the other hand, we prepared the acids from sphingosine and its dihydro derivative in larger quantities in order to permit a higher degree of purification.

As a result of this work it was found on one hand that the generally accepted melting point for tridecylic acid was much below the correct one, and the melting point of pentadecylic acid reported by us in the previous paper was higher than the correct one.

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* Received for publication, June 16, 1914.

[1] This *Journal*, xi, p. 547, 1912; xvi, p. 549, 1914.

The corrected melting point of the acids obtained from sphingosine and from its dihydro derivative, and also the melting points of their amides harmonize exactly with the corrected melting points of the corresponding normal acids and of their amides respectively. Hence, it becomes evident that the carbon atoms in the molecule of sphingosine are united in a normal chain.

An attempt was made to solve the configuration of the substituting groups by the action of ozone on sphingosine. It was expected, on the basis of the experience of Harries, to obtain by cleavage of the ozonide tridecylic aldehyde and an amino-tetrose according to the following reaction:

$$\begin{gathered}C_{17}H_{35}NO_2 + O_3 + H_2O = C_{12}H_{25}CHO + CH_2NH_2CHOH.CHOHCHO \\ + H_2O_2.\end{gathered}$$

For convenience of identification it was concluded to convert one into tridecylic acid and the other into tartaric acid. The tridecylic acid was isolated without great difficulty. The tartaric acid could not be isolated with certainty. In one experiment there formed a small quantity of crystals, which had the appearance of the calcium salt of mesotartaric acid. However, the quantity was insufficient for analysis, hence the conclusion regarding the configuration of the substituting groups will have to be deferred until future date.

EXPERIMENTAL PART.

The fatty acid obtained from the oxidation of sphingosine was again recrystallized from acetone, when it melted at 48–49°. Mixed with a sample of carefully purified tridecylic acid, it showed no depression of the melting point.

The acid was then changed into the amide by the usual method. Twice recrystallized from absolute alcohol it melted at 98–99°. The mother liquors gave a product, which recrystallized from 85 per cent alcohol, melted at 96°. This showed that the product was homogeneous. When mixed with the amide of normal tridecylic acid, the melting point was 97–98°. This leaves no doubt that the acid obtained by the oxidation of sphingosine is normal tridecylic acid.

Oxidation of Dihydrosphingosine.

About 30 grams of dihydrosphingosine were oxidized in 5-gram lots according to the directions given in the first paper. The green product obtained from the steam distillation was dried in ether solution, the ether removed and the product distilled in vacuum. We have tried to remove the chromium by heating with strong potassium hydroxide solution, but unsuccessfully. The colorless product obtained upon distillation was twice recrystallized from acetone. It melted at 53°.

0.5000 gram of substance required 20.0 cc. $\frac{N}{10}$ NaOH for neutralization.

	Calculated for $C_{15}H_{30}O_2$.	Found.
Mol. Wt.	242	250

The acid was then changed into the acid amide. This product, recrystallized from absolute alcohol, melted at 102° and when mixed with a sample of amide from normal pentadecylic acid, melted at 102–103°.

This establishes the constitution of the pentadecylic acid as the normal acid.

Action of Ozone upon Sphingosine.

Five grams of sphingosine sulphate were dissolved in 50 cc. of chloroform and the solution treated with ozone for a period varying from one to two hours. The preparation we used gave a red color in chloroform, more or less pronounced, and we found that when this color disappeared, that is when the solution became water-white, the reaction might be considered as ended. Under these conditions about one-eighth of the nitrogen was split off. If the ozone stream was continued longer, more and more of the amino nitrogen was destroyed.

The chloroform was distilled off the ozonide and the residue decomposed with water. It may be boiled for about ten minutes or shaken over night at room temperature.

The product was then extracted with ether. This removed the longer portion of the carbon chain. After removing the ether the product was taken up in acetone and oxidized with potassium permanganate. The acetone was filtered off, and the filtrate and precipitate worked up for fatty acid. The crude fatty acid was dissolved in methyl alcohol and neutralized to phenolphthalein with methyl alcoholic barium hydroxide. The precipitate was filtered off, decomposed with dilute hydrochloric acid, and reprecipitated several times. In some cases amyl alcohol was used to prevent foaming during the time of ozonizing. The valeric acid which resulted from the oxidation of this alcohol was washed out with hot water, and the acid finally purified through the lead salt. Recrystallized from acetone, the acid melted at 48–49°. One lot was characterized by changing it into the amide, which melted at 98–99°. This shows the acid to be normal tridecylic acid, as would be expected from the results of the first oxidation experiment.

0.5000 gram of substance required 23.2 cc. $\frac{N}{10}$ NaOH for neutralization.

	Calculated for $C_{13}H_{26}O_2$.	Found.
Mol. Wt.	214	215.5

The aqueous portion of the ozonide, after decomposition and extraction with ether, contained amino nitrogen, and reduced Fehling's solution. Theoretically one should expect in that solution the presence of an amino-tetrose. The isolation of this, or of a derivative of it, proved of great difficulty. Hence it was concluded to oxidize the aqueous part of the reaction product with nitric acid with the hope of obtaining tartaric acid. The aqueous solution, to which an equal part of strong nitric acid (sp. gr. 1.5) was added, was kept over night at 40°C, and the reaction product then rapidly evaporated on the water bath. In order to enhance evaporation, the operation was carried out in large watch glasses and in very small portions. The residues were taken up in water, boiled with calcium carbonate, allowed to cool and filtered. The filtrates on addition of ammonia water formed a precipitate. This was soluble in dilute hydrochloric acid and could be reprecipitated by ammonia. Once the precipitate formed in this manner had the calcium content of calcium tartarate.

0.1000 gram of substance, dried in xylol bath over phosphorous pentoxide and under diminished pressure, gave 0.0298 gram CaO.

	Calculated for $C_4H_4O_6Ca$.	Found.
CaO	29.7	29.8

It was then attempted to purify another sample prepared in the same manner following the directions of Anschütz.[2] On long standing there appeared a crystalline sediment. The crystals had the typical appearance of the calcium salt of mesotartaric acid, but the quantity was too small to permit an analysis.

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[2] *Ann. d. Chem.*, ccxxvi, p. 191, 1884.

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THE CULTIVATION OF HUMAN TISSUE IN VITRO.*[1]

BY JOSEPH R. LOSEE, M.D., AND ALBERT H. EBELING.

(From the Laboratories of the New York Lying-In Hospital and of The Rockefeller Institute for Medical Research.)

PLATES 75 TO 77.

The present investigations were undertaken to ascertain whether human connective tissue taken from a fresh cadaver could be kept in a condition of permanent life outside of the organism. We have applied to human tissues the method by which Carrel was able to keep animal connective tissue alive *in vitro* for more than two years.[2]

The first attempt to cultivate human tissues *in vitro* was made in 1911 by Carrel and Burrows.[3] Small fragments of human malignant tumors were placed in human plasma and incubated. In a few cases the fragments were surrounded after a few days by many cells; but generally liquefaction of the medium occurred and no growth was observed. In other experiments undertaken later by Carrel on the growth of normal tissues the same phenomenon was observed. Fragments of thyroid gland and fragments of connective tissue from adult individuals or from fetuses were inoculated into culture media which contained or did not contain tissue extract. The liquefaction was observed in this case in the same manner as in the case of the malignant tumors, and with the exception of a few

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* Received for publication, April 4, 1914.

[1] These investigations were carried on in March, 1913, in the research laboratory of the New York Lying-In Hospital. They were rendered possible by the coöperation of Dr. Markoe, of the attending surgeons of the Lying-In Hospital, and of Dr. Carrel. The Directors of the Hospital provided a specially equipped laboratory for the work, under the supervision of Dr. Markoe and Dr. Carrel, thus enabling us to carry out the study of the tissues of human cadavers under excellent conditions.

[2] Carrel, A., *Jour. Exper. Med.*, 1913, xviii, 287.

[3] Carrel, A., and Burrows, M. T., *idem*, 1911, xiii, 387.

cultures no real growth was observed. In 1913 Maccabruni[4] cultivated in plasma the tissues of the uterus and ovary of human fetuses and observed around the tissue after 8 or 9 days the presence of many cells and also of karyokinetic figures. The result of these experiments showed that human tissue almost always produced liquefaction of the plasma, and that this probably prevented its growth. We, therefore, attempted to develop a technique which would permit us to keep human tissue in a plasmatic medium without the occurrence of liquefaction.

At first we attempted to obtain a medium which would not liquefy under the influence of the tissue. The first medium tried was composed of human plasma and extract of human tissue taken from fresh cadavers. This, however, proved unsuitable, as in twenty-four hours liquefaction occurred about the fragments of tissue. Very little growth was observed, but when it took place the cells were seen to be scattered and the new growth to be very thin. A thin film containing many cells adhered to the cover-glass. When attempts were made to extirpate the film and to transfer it to fresh medium it generally became folded up and after one or two passages no further growth occurred. Many modifications of the medium were tried, in order to overcome these difficulties. Human plasma was diluted with agar, serum, and egg albumen. The addition of agar and egg albumen to the plasma yielded a firmer medium. No liquefaction took place, but on the other hand no real growth occurred. When the plasma was diluted with serum complete liquefaction rapidly followed. The addition of a small amount of acid or alkali to the plasma did not prevent this liquefaction. Finally after many attempts had been made it was found that by diluting the plasma with equal parts of Ringer solution a medium could be obtained which would not liquefy in less than 24 hours, and often not in 48 and 72 hours. Usually 18 hours after the medium had been inoculated with human tissue, growth appeared and increased progressively. After a period of from 24 to 96 hours the fragments of tissue were transferred to a fresh medium in which the growth continued. The medium was again modified by the addition of a small quantity of diluted extract of human tissue, after which the growth became very active. Finally at the end of 1913 and during the first months of 1914 it became possible to obtain large growths of human connective tissue. The tissue could be transferred from medium to medium; the new cells could be isolated from a primitive fragment and cultivated in a new medium; the mass of newly formed tissue rapidly increased in the same way that was observed by Carrel to occur in the case of the strain of connective tissue cells taken from the chick embryo, which are still proliferating at The Rockefeller Institute. Nevertheless, the technique which we have developed is still far from perfect. Liquefaction of the medium occurs as soon as the tissue extract becomes too concentrated. Besides, it appears that the plasma taken from many different individuals varies widely, the plasma of certain persons liquefying much more rapidly than that of others. The value of the extracts varies also, the result being that the medium is inconstant; and for this reason the rate of growth of fragments of tissue shows marked fluctuations in the course of a few weeks. The medium will probably be still further modified; nevertheless we shall describe here the technique that has rendered possible the cultivation of human tissue *in vitro*.

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[4] Maccabruni, F., *Ann. d. ostet. e ginecol.*, 1914, xxxvi, 57.

TECHNIQUE.

Plasma.—Blood is aspirated from the median basilic vein of adults under aseptic conditions with a dry, chilled, 10 c.c. glass syringe (gauge of needle, 19). The blood is immediately transferred to cold paraffined glass tubes and centrifuged in ice at high speed (3,000 revolutions) for 5 minutes. The plasma is at once pipetted off with a paraffined pipette into another cold paraffined tube, which was kept on ice ready for use. Several paraffined pipettes are prepared with bulbs to be kept in readiness, because it has been found that although the plasma remains liquid for some time in the paraffined tube it coagulates in the pipette used for making the cultures in from two to ten minutes. It is usually necessary to use a fresh pipette after changing two or three cultures.

Extract.—The extract is prepared from tissues taken from adult or fetal cadavers, and cut into small pieces. Equal parts of Ringer solution are added, the whole is placed in cold storage for 48 hours, after which it is centrifuged and the supernatant fluid pipetted off. This fluid may then be kept for some time in cold storage.

Making of Cultures.—Pieces of the various fetal tissues taken from fresh fetal cadavers are placed in Ringer solution or in serum and cut to the proper size with a cataract knife. The plasma is first spread on the cover-glass, and equal parts of Ringer solution are added and thoroughly mixed with it. The object of this procedure is to obtain a clot which can adhere firmly to the cover-glass, because it was found that when the plasma was diluted with the Ringer solution first coagulation took place too rapidly, thereby preventing a thorough embedding of the tissue. On the other hand, when the Ringer solution is spread first and the plasma added afterwards the clot does not adhere firmly, often contracting to such an extent as to become separated from the cover-glass, thereby destroying the culture. Then the tissue is put into the medium and the extract immediately added. The medium usually coagulates about the piece within a few seconds. A reasonable amount of speed and strict aseptic precautions are observed throughout. The preparations are next placed over a concave slide hermetically sealed with paraffin and incubated at 38° C., and the tissues allowed to grow. When a fresh amount of growth has taken place after a period varying from 24 to 96 hours, the tissues are removed from their medium, washed, and transferred to a fresh medium. The time of the transfer depends upon the condition of the medium and the activity of the growth. Observations are taken at intervals of 12, 24, 48, and even 96 hours. Observations are likewise made of cultures fixed and stained with hematoxylin and with Giemsa stain.

RESULTS.

With the technique described above, it became possible to keep a strain of connective tissue cells, derived from a piece of skin which was obtained from a fresh four months old fetal cadaver, in a condition of active life *in vitro* for more than two months.

When the fragments of fetal heart tissue, obtained from fresh cadavers, were introduced into a medium composed of equal parts of human plasma and Ringer solution, and incubated at 38° C. for 16 hours, microscopic examination of the culture showed the fragments as sharply outlined masses in a slightly opalescent medium with no evidences of cell proliferation. In from 18 to 24 hours cell proliferation manifested itself, in some cases by the appearance of many cells spreading out or budding from the original piece; in other cases by the presence of only a few scattered cells.

The rate and extent of growth varied and probably depended on the vitality of the tissue, the constituents of the medium, and the changes that developed in it. Where there was a tendency for the proliferating cells to grow in one or two planes the growth was greater and more rapid than where the cells appeared to grow in several planes. In the latter case the growth was also more dense.

The phenomenon of growth was accompanied by a progressive and slow liquefaction of the medium around the primitive fragment and in the zone of cell proliferation. Sometimes liquefaction around the entire fragment took place within 24 hours after the cultures were made, although little, if any, growth was apparent. At times the medium retracted from the piece in certain areas and the vacuoles in the medium thus produced contained fluid in which detached cells and débris were observed. The liquefaction did not indicate necrosis of the piece of tissue in all instances. Pieces around which the medium liquefied completely in 24 hours have been transplanted into fresh medium, in which cell proliferation took place in from 16 to 24 hours. There was no evidence of rapid liquefaction.

When liquefaction was apparently due to cell proliferation, it started from the periphery of the fragment and was progressive. On the other hand, when apparently due to substances in the medium it was general and affected the entire mass of the medium. Liquefaction of the entire medium took place when extract from fetal tissues was used in primitive cultures. Primitive fragments grew well without the addition of extract to the medium for one or two passages, but usually after the second passage it was necessary to add extract in order to stimulate growth. The time of the passages into fresh medium was governed by the condition of the culture. Where the medium showed liquefaction around the primitive piece in 24 hours, a change was made. If the rate of growth was rapid, the culture was changed after 48 hours. Actively growing cultures which were not changed until after 72 hours grew less well. In cases where the time of passage was deferred for 96 hours, subsequent passages showed a decided retardation in the rate and extent of growth of the cells and the cellular characters were altered. Ameboid cells occurred and rapid liquefaction of the entire medium often took place; and although passages into fresh medium were made every 24 or 48 hours the cultures did not recover.

In the history of one culture, which is given in table I, it will be noticed that 24 hours after the 33d passage excellent growth was recorded; 96 hours later the culture was changed, and in 24 hours observation showed retarded cell proliferation with subsequent liquefaction. After the 34th passage, that is, 68 days, there was no further growth and death of the culture gradually resulted. In the

TABLE I.

Passage.	Date (1913).	Treatment of culture.	Observations.
Experiment 121-1	Oct. 21	Culture of skin from a 4 months' fetal cadaver cultivated in 1 drop of plasma and 1 drop of Ringer solution	Oct. 22. Medium in good condition; no growth. Oct. 23. Medium in good condition; growing. Oct. 24. Medium slightly liquefied; growth continuing.
1	Oct. 24	Washed in Ringer solution for 1 minute and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 drop of extract	Oct. 25. Medium liquefied; no growth.
2	Oct. 25	Washed in Ringer solution for 1 minute and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 small drop of extract	Oct. 26. Medium in good condition; growing. Oct. 27. Medium partially liquefied; growing.
3	Oct. 27	Washed in Ringer solution for ½ minute and cultivated in the same medium	Oct. 28. Medium slightly liquefied; growing. Oct. 29. Medium liquefied; growing.
4	Oct. 29	Washed in Ringer solution for ½ minute and cultivated in 2 drops of serum, 1 drop of plasma, and 1 small drop of extract	Oct. 30. Medium completely liquefied; some growth.
5	Oct. 30	Washed in Ringer solution for 1 minute and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 small drop of extract	Oct. 31. Medium in good condition; excellent growth. Nov. 1. Excellent growth.
6	Nov. 1	Divided into 2 pieces, washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 3. Medium in good condition; excellent growth.
7	Nov. 3	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 4. Medium in good condition; excellent growth. Nov. 5. Excellent growth.
8	Nov. 5	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 6. Medium in good condition; excellent growth (macroscopic). Nov. 7. Excellent growth.
9	Nov. 7	Divided into 2 pieces, washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 8. Medium in good condition; growing. Nov. 9. Good growth.
10	Nov. 9	Cut into 2 pieces, washed in Ringer solution for ½ minute, and cultivated in the same medium	Nov. 10. Medium in good condition; excellent growth. Nov. 11. Medium in good condition; excellent growth.
11	Nov. 11	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 12. Medium in good condition; growing well.
12	Nov. 12	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 13. Medium in good condition; good growth.

TABLE I.—*Continued.*

Passage.	Date (1913).	Treatment of culture.	Observations.
13	Nov. 13	Culture washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 14. Growing. Nov. 15. Good growth.
14	Nov. 15	Partially divided, washed in Ringer solution, and cultivated in the same medium	Nov. 17. Good growth.
15	Nov. 17	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 18. Good growth. Nov. 19. Excellent growth.
16	Nov. 19	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Nov. 20. Medium slightly liquefied; growing. Nov. 21. More liquefaction of medium; excellent growth.
17	Nov. 21	Washed in Ringer solution for ½ minute and cultivated in the same medium	Nov. 22. Excellent growth.
18	Nov. 22	Washed in Ringer solution for ½ minute and cultivated in the same medium	Nov. 23. Good growth. Nov. 24. Growing.
19	Nov. 24	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 25. Very good growth. Nov. 26. Liquefaction; excellent growth.
20	Nov. 26	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Nov. 27. Excellent growth. Nov. 28. Liquefaction; excellent growth.
21	Nov. 28	Divided into 2 pieces, washed in Ringer solution for 1 minute, and cultivated in the same medium	Nov. 29. Very good growth.
22	Nov. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 30. Good growth. Dec. 1. Liquefaction; excellent growth.
23	Dec. 1	Washed in Ringer solution for ½ minute and cultivated in the same medium	Dec. 2. Excellent growth (dense).
24	Dec. 2	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 3. Good growth (dense). Dec. 4. Good growth (dense).
25	Dec. 4	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 5. Good growth. Dec. 6. Excellent growth.
26	Dec. 6	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Dec. 7. Growing slowly. Dec. 8. Liquefaction and growing slowly. All cultures this day liquefied.
27	Dec. 8	Washed in Ringer solution for 45 seconds and cultivated in the same medium	Dec. 9. Growing. Dec. 10. Slight liquefaction; growing.
28	Dec. 10	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 11. Growing. Dec. 12. Growing.
29	Dec. 12	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 14. Good growth. Dec. 15. Excellent growth.

TABLE I.—*Concluded.*

Passage.	Date (1913).	Treatment of culture.	Observations.
30	Dec. 15	Central piece extirpated and the remaining cells cultivated in 1 drop of plasma and 1 drop of extract	Dec. 16. Excellent growth. Dec. 17. Excellent growth.
31	Dec. 17	Washed in Ringer solution and cultivated in 1 drop of plasma, 1 drop of Ringer solution, and 1 drop of extract	Dec. 18. Growing very well. Dec. 19. Growing very well.
32	Dec. 19	Divided into 2 pieces, washed in Ringer solution for 1 minute, and cultivated in the same medium	Dec. 20. Liquefaction; growing. Dec. 21. Liquefaction; excellent growth.
33	Dec. 22	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 22. Excellent growth. Dec. 23. Growing. Dec. 24. Medium in good condition; no liquefaction. Dec. 25. Medium in good condition; no liquefaction. Dec. 26. Medium in good condition; no liquefaction.
34	Dec. 26	Divided into 2 pieces, washed in Ringer solution for 1 minute, and cultivated in the same medium	Dec. 27. Growing slowly. Dec. 28. Growing slowly. Dec. 29. Liquefaction; growing slowly.
35	Dec. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 30. No growth; ameboid cells. Dec. 31. Complete liquefaction.
36	Dec. 31	Culture washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 2, 1914. Few cells, mostly ameboid in character.

first and succeeding passages growth was more extensive in a given time than that which develops in a primitive culture; also a definite relation between the extent of growth and the degree of liquefaction which is produced by the growth was suggested.

Fluctuations were observed in the rate and extent of growth after passages, a point which is shown in the history referred to. This fluctuation affected all cultures which were changed at the same time and into the same medium. The probable causes are the differences in various specimens of plasma and the effect of the extract employed on them. In successive passages the primitive fragment gradually disappeared; and on extirpation of the primitive fragment the peripheral cells after being washed and cultivated in fresh medium continued to proliferate and form a network of tissue as shown in figure 1. Figure 2 shows the extirpated fragment which was washed and cultivated and incubated for forty-eight hours, after which it was stained.

In actively growing cultures the cells appeared as delicate spindle-shaped, fusiform bodies, isolated or united by filaments sometimes closely assembled, forming a network which was either film-like or quite dense, depending upon the tendency of the cellular outgrowths. The cytoplasm appeared granular and the nucleus stood out as a clear, homogeneous area in which mitotic figures were distinguishable. Refractile globules were to be seen in the cytoplasm of the cells. Their size and number varied. The longer a culture was left unchanged the larger and more numerous were these globules. When conditions in the culture were unfavorable, the cells presented ameboid characteristics, and there was no tendency towards the formation of a cellular network. Refractile fat globules then became frequent in the cell cytoplasm.

When the cultures were fixed and stained with Giemsa stain the outline and structure of the cells became distinct. The cytoplasm appeared as a light blue, finely granular body with a more deeply stained, coarsely granular nucleus. The structures containing chromatin took on a deep purple stain. Figure 3 represents a photograph of stained individual cells highly magnified.

Although growth of human connective tissue derived from fetal heart tissue has been obtained which was as extensive as the growth of connective tissue derived from embryonic heart tissue of the chick, the growth of human tissue was usually less dense and less extensive. Fluctuations which occurred during the passages of human cultures were greater than those occurring in cultures of chick tissue. Liquefaction in human tissue cultures was more marked and eventually interfered with cell proliferation. In cultures of chick tissue the liquefaction that took place was slight and did not interfere with cell proliferation.

The fetal human tissue which was used in making cultures was generally obtained from fetal cadavers 4 to 6 months old; the embryonic chick tissue was obtained from 8 to 15 day old embryos. The latent period where human tissue was cultivated was usually from 16 to 18 hours. For chick tissue growth appears within 10 or 12 hours after the original fragment has been embedded.

SUMMARY.

A strain of human connective tissue was kept in a condition of active life *in vitro* for more than two months. When a medium has been devised the composition of which is more constant, it is reasonable to suppose that human connective tissue can be cultivated *in vitro* for an indefinite period.

EXPLANATION OF PLATES.

PLATE 75.

FIG. 1. Human connective tissue cells, fixed and stained with Giemsa stain. The culture was made by extirpating the central portion of culture 285 in its 16th passage, washing the remaining portion of the culture with Ringer solution without removing it from the cover-glass, and dropping on fresh plasma and extract. The preparation shows the extent of growth obtained in 48 hours from peripheral cells remaining after extirpation of the fragment.

PLATE 76.

FIG. 2. 17th passage of human connective tissue, fixed and stained with Giemsa stain. The photograph shows the extent of growth obtained after passage into fresh medium. The fragment in this culture was the piece extirpated from culture 285.

PLATE 77.

FIG. 3. 17th passage of human connective tissue cells. Individual cells; high power magnification. Some of the cells are shown in figure 2.

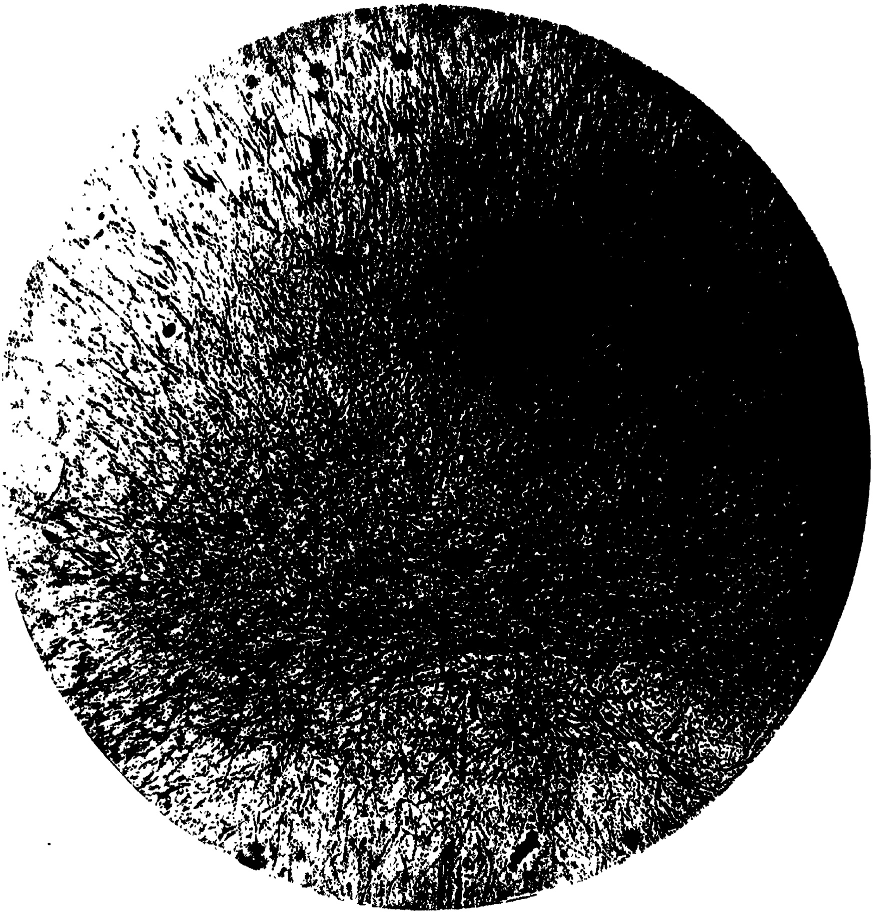
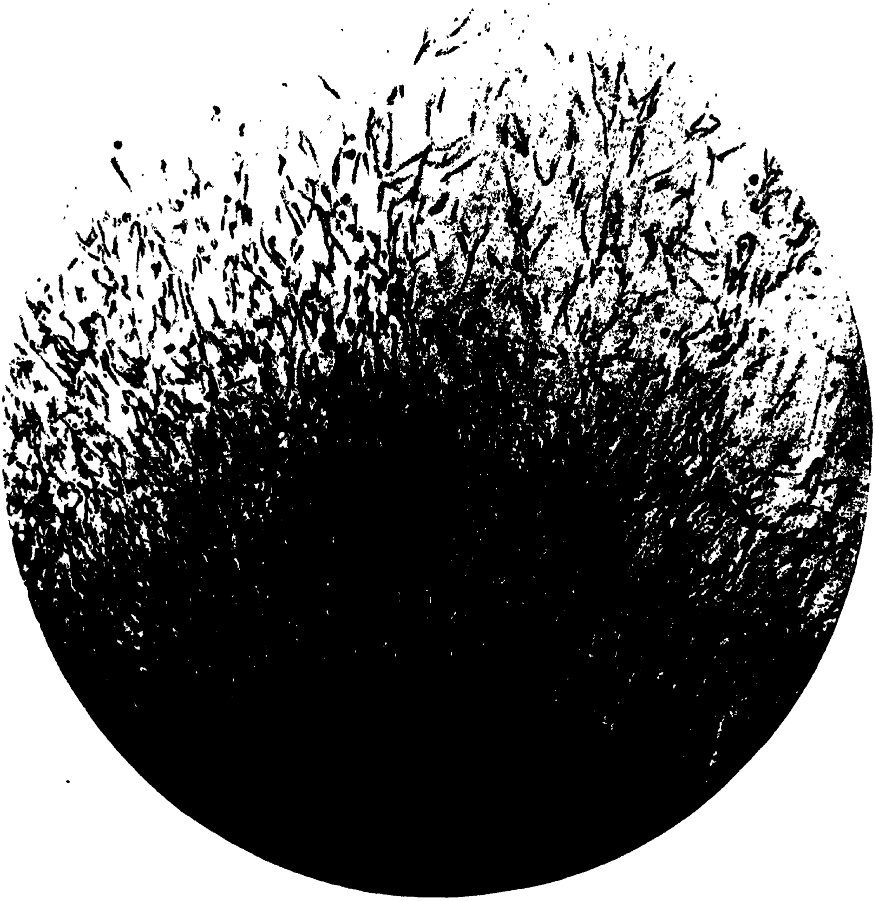


FIG. 1.

(Losee and Ebeling: The Cultivation of Human Tissue *in vitro*.)



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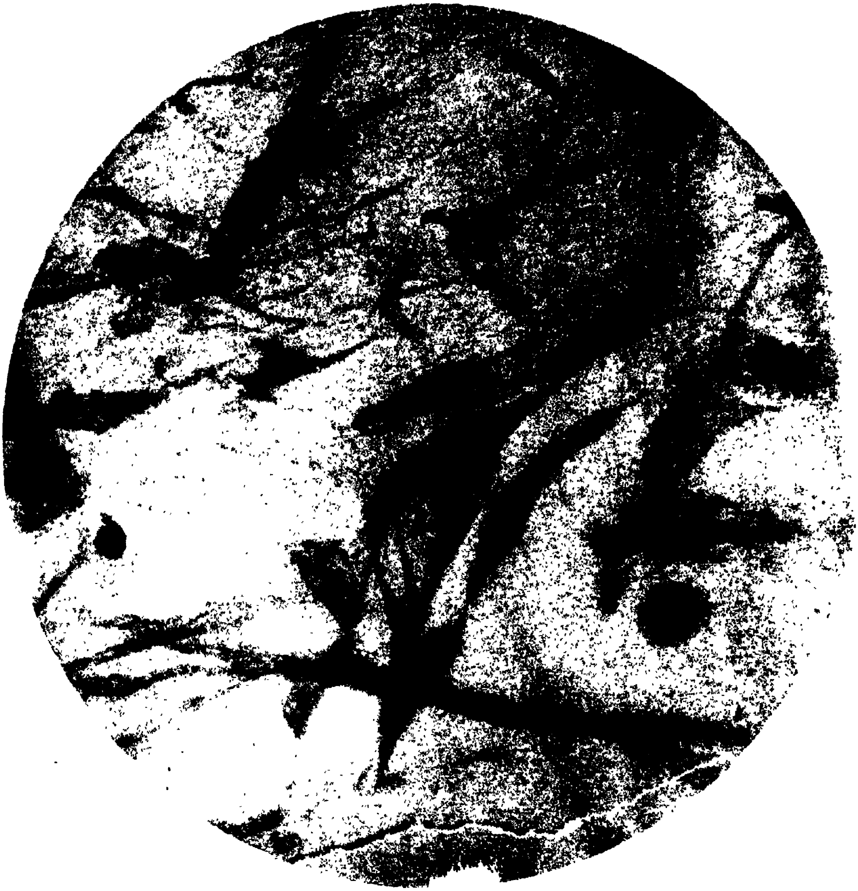

(Ebeling. The Cultivation of Human Tissue *in vitro*.)

[Reprinted from The Journal of Experimental Medicine, July 1, 1914, Vol. xx, No. 1, pp. 1–2.]

PRESENT CONDITION OF A STRAIN OF CONNECTIVE TISSUE TWENTY-EIGHT MONTHS OLD.*

By ALEXIS CARREL, M.D.

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Plates 1 and 2.

In previous articles[1] it was shown that connective tissue could be kept outside of the organism in a condition of permanent life. The purpose of the following experiments was to determine the present condition of a strain of connective tissue which, after having undergone 358 passages, has now reached the twenty-ninth month of its life *in vitro*. The strain of connective tissue was derived from a piece of heart extirpated on January 17, 1912, from a chick embryo seven days old. The fragment of heart pulsated for 104 days and gave rise to a very large number of connective tissue cells. These cells multiplied actively during the last two years, and produced a large amount of connective tissue. At present, a great many cultures are obtained from the strain every week.

The dynamic condition of a tissue is manifested by the rate of its growth. The increase in the volume of a fragment of connective tissue can be measured with comparative accuracy. For this the following technique is used. A fragment of tissue is removed from a culture, washed in Ringer solution, and placed in a new medium. It soon becomes surrounded with a ring of new tissue. After forty-eight hours the width of this ring is measured with a micrometer. Under the ordinary conditions of the experiment the thickness of the new tissue is more or less uniform, and its total volume can therefore be estimated fairly accurately by its superficial size. The fragments of tissue usually double in forty-eight hours. But their rapidity of growth is subject to fluctuations dependent upon the character of the medium and upon the condition of the tissue at the time that it is placed in the medium. When these conditions were favorable the ring of new tissue attained, during

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* Received for publication, May 10, 1914.

[1] Carrel, A., *Jour. Exper. Med.*, 1912, xv, 516. Ebeling, A., *idem*, 1913, xvii, 273. Carrel, A., *idem*, 1913, xviii, 287.

the last few months, a width of 2 to 2.8 millimeters (figures 1 and 2). A comparison of the amount of tissue produced by a given culture in forty-eight hours this year with that produced in the same time by the same strain of cells a year ago shows that the activity of the strain has increased. Last year the width of the ring of tissue produced in forty-eight hours around the fragments of connective tissue was only 1.5 or 1.8 millimeters.

This increase in the rate of growth is made more apparent by the following experiment. A piece of heart extirpated from a chick embryo eight days old, and a fragment of connective tissue at the beginning of the third year of its life *in vitro* were placed in the same culture medium. After forty-eight hours it was seen that the tissue which had become adapted to the life *in vitro* had increased much more rapidly than the fresh tissue (figure 3). Nevertheless, the tissue adapted to the life *in vitro* was derived indirectly from a fragment of heart extirpated more than two years ago from an embryo seven days old. Thus it is conclusively shown that the proliferating power of the strain has in no wise diminished. Nevertheless, it would be imprudent to conclude from this fact that it has augmented, as the greater rate of increase of the tissues may be due, not to an augmentation of the proliferating power of the cells, but to an improvement in the details of the technique.

Moreover, the fact remains that during the third year of independent life the connective tissue shows greater activity than at the beginning of that period, and is no longer subject to the influence of time. If we exclude accidents, connective tissue cells, like colonies of infusoria, may proliferate indefinitely.

EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. A fragment of connective tissue extirpated from a culture of the twenty-eight months old strain, one hour after the passage.

FIG. 2. The same tissue, forty-eight hours after the passage.

PLATE 2.

FIG. 3. In the same medium were placed a piece of heart from a chick embryo eight days old (A), and a fragment of connective tissue, No. 8860, which had lived for more than two years outside of the organism (B). The photograph shows the amount of tissue produced in forty-eight hours by both fragments.

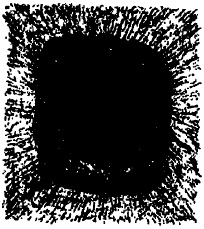

FIG. 1.

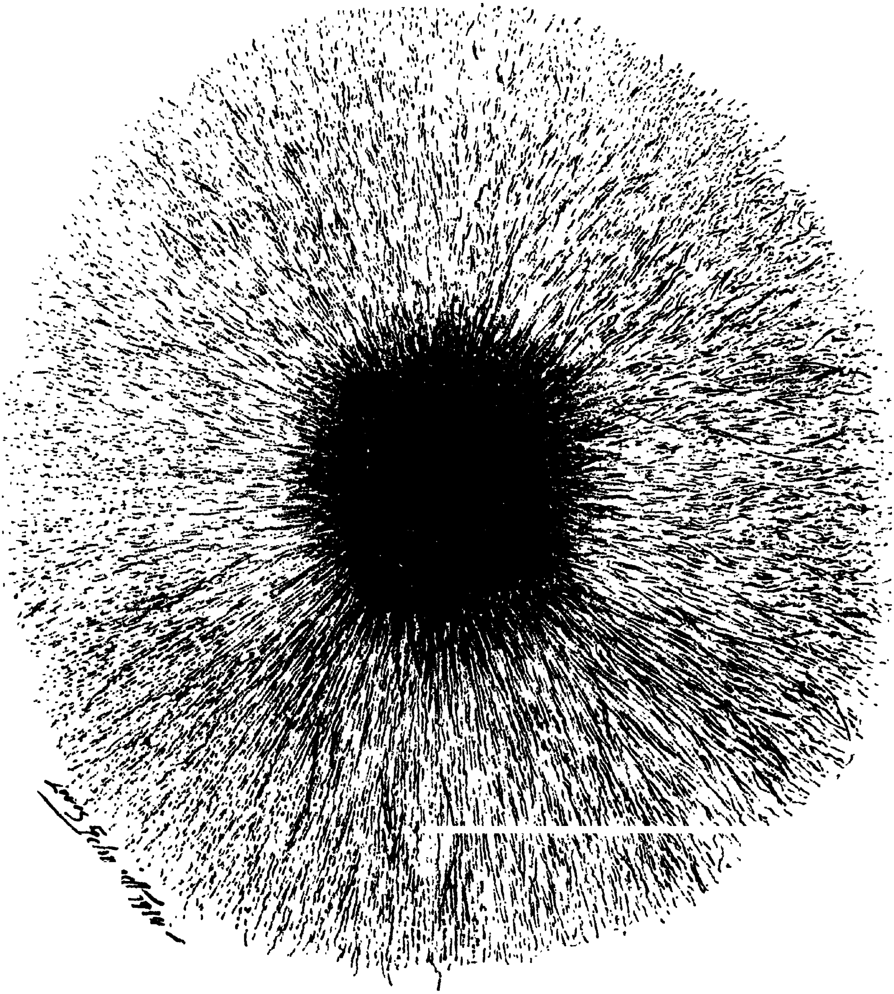

FIG. 2.

(Carrel: Condition of Strain of Connective Tissue.)

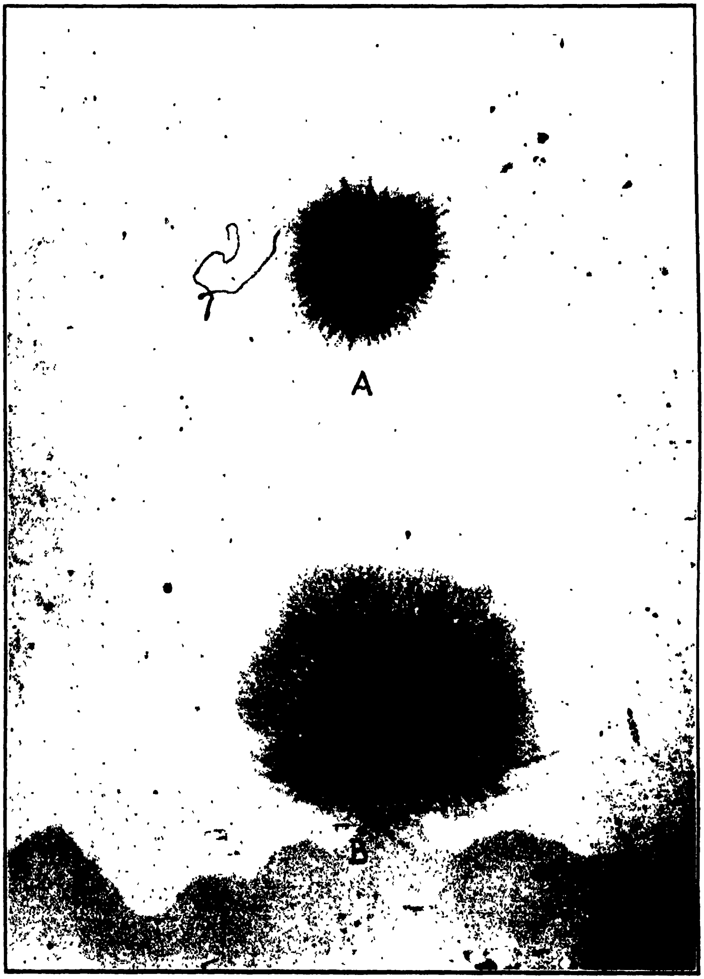

FIG. 3.

(Carrel: Condition of Strain of Connective Tissue.)

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PATCHING AND SECTION OF THE PULMONARY ORIFICE OF THE HEART.*

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

The purpose of these experiments was to develop a technique by means of which the pulmonary orifice could be enlarged. The operation consisted in suturing to the anterior side of the orifice a venous patch which permitted an increase in the circumference of the orifice after the arterial wall had been incised.

TECHNIQUE.

The animals were etherized according to the Meltzer-Auer method. The thoracic wall was shaved with sodium sulphide and sterilized with iodin, and the animal was put on an electric warming pad. The thoracic cavity was then opened on the left side by a large transverse incision which was kept widely open by means of a Gosset retractor. The operating field was walled off by means of a technique precluding the occurrence of pleurisy, which has already been described.[1] The pericardium was opened through a long incision made on the right side of the phrenic nerve at about two centimeters' distance from the nerve, and the anterior part of the heart and of the pulmonary artery was exposed.

The position of the valves was easily located. The wall of the pulmonary artery is thinner at the level of the sigmoid sinuses and its color is slightly darker. This point was selected for the lower end of an incision of about 1.5 centimeters made through the arterial wall above the anterior sigmoid valve. It was found afterwards that this incision was too short, and that when it was longer it produced a very marked pulmonary insufficiency. In order to be more efficient and less dangerous, the section of the orifice should be

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* Received for publication, May 10, 1914.

[1] Carrel, A., *Surg., Gynec. and Obstet.*, 1914, xix, (in press).

made at the point of insertion of the anterior and left sigmoid valves on the arterial wall. After the location of the incision had been selected the patch was applied on the anterior wall of the pulmonary artery and of the heart. This patch was usually a piece of vena cava which had been preserved in cold storage for a few days. Pieces of artery could also be used, but as the arterial wall is thicker and harder than the venous wall it was more difficult to fix the patch to the pulmonary wall without danger of leakage. The shape of the patches was generally rectangular, about 2.5 by 2.5 centimeters. The patch was fixed to the pulmonary artery and to the heart by four stitches. The stitches of the lower and upper sides of the rectangle were about 2.5 centimeters distant from each other. The right and left sides of the patch were placed in such a way that the distance between the right and left lines of suture was only about 1.5 centimeters. As the width of the flap was 2.5 centimeters this allowed of one centimeter's increase in the circumference of the pulmonary orifice after it had been cut. The right upper and left sides of the flap were fixed to the anterior wall of the pulmonary artery by a continuous suture. Between the lower end of the flap and the wall of the heart there was a free space through which the incision of the orifice could be made. For this purpose special scissors were constructed, composed of one long sharp blade and one short blunt blade. The arterial wall was perforated with the long blade of the scissors just at the level of the pulmonary orifice. The long blade was pushed into the lumen of the pulmonary artery, and the wall was cut by introducing the short blade deep under the flap. There was an immediate escape of dark blood, the scissors were quickly removed and at the same time the index finger of the assistant compressed the flap against the opening made with the scissors, thus arresting the hemorrhage. Then the lower side of the flap was rapidly united to the cardiac wall by means of a continuous suture. This stage of the operation was generally made without interrupting the circulation through the heart. The operation was facilitated by interrupting it for one or two minutes; but as the clamping of the pedicle of the heart is always somewhat dangerous, this procedure was not often used. After the suture had been completed slight compression with a sponge was applied on the flap and on the lines of suture. If there was still some leakage after a few minutes, complementary stitches were added. The pericardium was never closed before the line of suture was absolutely secure against the escape of blood. After suture of the pericardium, the thoracic cavity was closed by the ligation of two ribs and by three or four planes of suture. Afterwards the dog was dressed and cared for by the customary methods.

EXPERIMENTAL.

PATCHING OF THE PULMONARY ARTERY.

The experiments were performed on eight medium sized dogs. The technique for the first four operations was in its developmental stage and differed slightly from that used for the last four operations.

Experiment 1.—Black female dog, medium size. Oct. 23, 1913. Etherization by the Meltzer-Auer method. Transverse thoracotomy. Ligature of both mammary arteries and section of the sternum. Opening of both pleural cavities. Incision of the pericardium. On the anterior part of the pulmonary artery and of the cardiac wall was placed a fragment of aorta taken twenty-four hours previously from a fresh cadaver of a human fetus and preserved in cold storage. The upper and the lateral sides of the patch were fixed to the pulmonary wall by a continuous suture. Then the pedicle of the heart was clamped. A small knife was introduced under the patch and the wall of the pulmonary artery was cut. The incision was too short and too high. Next, the lower side of the patch was sutured to the cardiac wall, and the clamp was removed after the circulation had been interrupted for two minutes. Massage of the heart. After a few minutes the contractions were normal. Closure of the pericardium and of the thoracic cavity. No shock. After the operation the animal remained in normal condition. May 5, 1914. Animal in excellent health.

Experiment 2.—White dog, medium size. Oct. 26, 1913. Etherization by the Meltzer-Auer method. Opening of the thoracic cavity by the same method as that used in experiment 1. The patch was made of a fragment of dog's aorta preserved for twenty-four hours in cold storage. The clamping of the pedicle of the heart and the suture of the flap were performed as in the previous operation. The section of the wall of the pulmonary artery was made with fine scissors at the level of the insertion of the sigmoid valves. After the clamp was removed fibrillary contractions appeared and the animal died. An examination of the specimen showed that the wall of the pulmonary artery was cut as far as the insertion of the anterior valve. The opening was completely protected by the patch, but a small branch of the coronary artery had been taken up by a stitch. This was possibly the cause of the fibrillary contractions. The incision should have been made further to the left side of the artery, in order to cut the orifice at the point of insertion of the left and anterior valves.

Experiment 3.—Black and white bulldog, medium size. Oct. 31, 1913. Etherization by the Meltzer-Auer method. Transverse thoracotomy on the left side without section of the sternum and without ligation of the mammary arteries. A piece of dog's jugular vein preserved for twenty-four hours in cold storage was placed on the pulmonary artery. The lateral and the lower sides were fixed to the arterial and cardiac wall by a continuous suture. Then the wall was cut with scissors as far as the insertion of the sigmoid valves. Hemorrhage was prevented by compressing the flap to the opening with the index finger. Next, the upper side of the flap was sutured to the arterial wall. No massage of the heart. Normal pulsations. Closing of the pericardium and of the thoracic cavity. Two hours afterwards the animal was in excellent condition. May 5, 1914. Dog normal.

Experiment 4.—Female bulldog, medium size. Nov. 3, 1913. The same technique was used as in experiment 3. The patch was taken from a piece of vena cava preserved in cold storage since Oct. 27. It was sutured without the heart being clamped. No shock. The animal remained in good condition after the operation. Nov. 6. Animal ill. Nov. 8. Animal died.

Autopsy.—Purulent pericarditis. The space between the flap and the arterial wall was filled by a clot. The incision had healed. No fibrin or thrombus in the lumen of the vessel. As the incision was too short there was no increase in the circumference of the orifice.

Experiment 5.—Black and white male fox-terrier. Nov. 4, 1913. Etherization by the Meltzer-Auer method. Opening of the thoracic cavity and the pericardium with the same technique as that used in experiment 4. The patch was made from a piece of vena cava preserved in cold storage since Oct. 27. It was fixed on three sides and the anterior wall was opened with a cataract knife without any interruption of the circulation of the heart. Then the fourth side was sutured and the operation completed by the ordinary method. After the operation the dog remained in excellent condition. On Nov. 19 the animal had completely recovered. Strong diastolic murmur could be heard. May 1, 1914. Animal in excellent condition. The murmur had almost completely disappeared.

Experiment 6.—White female bulldog. Nov. 11, 1913. The technique used was identical with that of experiment 5. On Nov. 19 no murmur was audible. The animal remained in excellent condition, and is completely normal on May 5, 1914.

Experiment 7.—White male bulldog. Nov. 26, 1913. Etherization by the Meltzer-Auer method. The technique was identical with that used in the last two experiments. The section of the wall was made with special scissors composed of a long sharp blade and a short blunt one. The animal remained in excellent condition after the operation and is still normal on May 5, 1914.

Experiment 8.—White female bulldog. Dec. 2, 1913. Etherization by the Meltzer-Auer method. Technique identical with that used in the last three operations. The patch was made from a piece of jugular vein preserved in cold storage since Nov. 28. The animal remained in excellent condition and is still normal on May 5, 1914.

RESULTS.

The results of these operations must be considered from two standpoints: the danger to the life of the animal, and the modifications of the pulmonary orifice.

When the technique previously described was carefully followed, the operation was of little danger to the life of the animal. Although these operations were in the developmental stage, nevertheless only two animals died of the eight that were experimented upon. One of these died on the operating table from fibrillary contractions, probably due to the fact that a branch of the coronary artery had been taken up by one of the stitches. A second dog died of pericarditis a few days after the operation. It is probable that neither of these complications will occur again, since a better way of handling the heart has been found. In the six other instances the animals sustained no shock after the operation, remained in good health, and were still normal more than six months after the operation. The operation will be much less dangerous in the future, because the details of the technique have now been completely established.

The changes produced in the anatomy of the pulmonary orifice were examined on two specimens. In experiment 2 the dog died of fibrillary contractions on the operating table. The specimen showed that the incision had been too short and was located on the right side of the artery. The operation as it was performed was insufficient as well as dangerous. It was insufficient because the circumference of the orifice was not increased; and it was dangerous because the flap was fixed to the cardiac wall at a site where there were many branches of the coronary arteries. In experiment 3 the animal died of pericarditis. The flap had taken, but the incision was too short. The examination of the flap and of the incision demonstrated that no thrombosis had taken place, and that the patch of vein in contact on one side with dark blood and on the other with the pericardium, although in an unfavorable condition of nutrition, did not undergo necrosis. In the clinical examination of the six other animals it was found that only one presented a diastolic murmur a few weeks after the operation; that is, in one instance only was pulmonary insufficiency obtained. The lack of insufficiency in the other cases was due to the fact either that the incision of the pulmonary artery was located too high and was too short, or that the flap was fixed too tightly to the arterial wall and did not allow of the dilatation of the orifice after it was incised. These details had to be learned in the course of the experiments, and it will be an easy matter to make an incision and patching which will permit a real dilatation of the orifice to take place. The incision must be about two centimeters in length, half being on the pulmonary artery and half on the ventricle, and it must cross the orifice at the point of junction of the left and the anterior sigmoid valves. The incision of the orifice and the suture of the fourth side of the flap will be made after the pedicle of the heart is clamped.

The six surviving animals are still in good health more than six months after the operation. In experiment 5 the diastolic murmur almost completely disappeared.

CONCLUSION.

These experiments show that it is possible to perform an operation, the object of which is to increase the circumference of the pulmonary orifice without involving much danger to the life of the animal. It is probable that operations of this type may come to be employed in the treatment of stenosis of the pulmonary artery in man.

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EXPERIMENTAL OPERATIONS ON THE SIGMOID VALVES OF THE PULMONARY ARTERY.*

BY ALEXIS CARREL, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

PLATES 3 TO 5.

The purpose of the following study was to ascertain whether and to what extent intracardiac operations could be performed with safety. The technique that has been described and employed by various experimenters and surgeons is unsatisfactory, because it involves the introduction of sharp instruments into the cavities of the heart, without the control of the eye or the finger. Thus far no operations have been devised which permit, for example, the cauterization of infected valves, the suture of the foramen ovale, or of two valves in a case of insufficiency, and still other plastic operations. The operations mentioned form a different class from those which have so far been performed, because they involve the stoppage of the circulation through the cavities of the heart and the passage of air into the cardiac cavities, and require great speed of execution. Hence it is hardly to be expected that a technique making possible and safe such operations as these will be perfected soon. It may even be regarded as extremely doubtful whether this class of operations may ever be applicable to human surgery. And yet their future cannot be predicted, and I have, therefore, attempted to develop methods for the execution of these operations. For the preliminary studies the sigmoid valves of the pulmonary artery were selected, because the opening of the right cavities of the heart is less dangerous than the opening of the left cavities.

METHODS.

The preparation of the animals is similar to that already described.[1] After the thoracic cavity has been opened in the anesthe-

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* Received for publication, May 20, 1914.

[1] Tuffier, T., and Carrel, A., *Jour. Exper. Med.*, 1914, xx, 3.

tized animal by a left transverse thoracotomy, the pericardium incised, and the anterior part of the heart exposed, the operation is started. The operation consists of four stages: (1) the stoppage of the circulation of the heart, (2) the opening of the pulmonary artery, (3) the performance of the intracardiac operation and the closing of the pulmonary artery, and finally (4) the reëstablishment of the circulation through the heart.

The Stoppage of the Circulation of the Heart.—This has already been performed in many different ways. We ourselves have used all known methods of stopping the circulation through the heart. Finally, we adopted the method of clamping in mass the pedicle of the heart by means of large soft-jawed forceps. The heart was not taken out of the pericardium, but the incision of the pericardium was large enough to permit of the easy introduction of one of the jaws of the forceps under the pedicle. The forceps was a Doyen, the jaws of which were covered with rubber. One of the jaws was introduced into the pericardium under the pedicle and directed from the right to the left side by the index finger of the operator. Before clamping, the position of the forceps was carefully examined. The handling of the forceps and of the pedicle was always very gentle. Care was taken not to produce any compression of the veins before the time of the clamping. It is important for the heart to be in normal condition before clamping the pedicle, a result obtained by overventilating the blood, which is easily accomplished by means of the Meltzer-Auer apparatus. It was noticed that if the heart is clamped before it is in excellent condition the interruption of the circulation is less safe than when the heart is filled with well oxygenated blood. The advantage of using the Meltzer-Auer method in this operation is that it permits of an acceleration of the oxygenation of the blood at will. When everything is ready for the operation the forceps is rapidly clamped and, without a second being wasted, the heart or the vessel is opened and the operation started.

When these precautions are taken it is possible to clamp the pedicle of the heart for as long as two and a half or three minutes without subsequent trouble. As soon as the clamp is removed the heart resumes pulsation, and after a short time the pulsations are again normal. In no case was there any need of massaging the heart when the interruption of the circulation did not exceed two and one half minutes; this period probably allows of a considerable margin of safety and it would doubtless be possible to prolong the operation slightly without excessive danger. But two and one half minutes appear to be sufficient time for the performance on the valves of several operations.

The above technique may appear to be somewhat crude, but experience has shown that it is simpler than the separate clamping of the arterial and venous pedicles, and requires less handling of the heart, which is an important consideration. Moreover, traumatism of the anatomical structures of the pedicle is slight, on account of the large quantity of tissue which is taken up between the jaws of the forceps and which renders their action less rough.

The Opening of the Pulmonary Artery.—The pulmonary orifice is exposed by means of an incision made through the anterior wall of the artery at the level of or a little above its junction with the heart. The incision is made with sharp scissors, or is begun with sharp scissors and finished with blunt scissors. The wall of the pulmonary artery is perforated about one and one half or two centimeters above the point of insertion of the sigmoid valves and incised from above downwards with one cut of the scissors. The location and the length of the incision vary according to the purpose. When the incision is located above the anterior valve it is not prolonged below the level of the insertion of the valve, in order not to incise the sigmoid. When the incision is made further on the left side of the artery at the level of the junction of the anterior and left sigmoids of the valve it can be prolonged further on to the cardiac wall, and an incision four centimeters in length, half on the heart and half on the pulmonary artery, could be made without danger. The short incision made above the orifice is kept open by two forceps put on the edges, when the valves are sufficiently widely exposed to be cauterized. But through such an opening it is impossible to perform a suture of the valves. Hence for these cases a long incision is made at the union of the anterior and left sigmoids (figures 1 and 2), which permits a large opening of the pulmonary artery and the possibility of operating easily on the valves themselves. It is not necessary to cut exactly at the point of insertion of the valves. If the incision is located near the point of insertion of the valves it does not produce a marked degree of insufficiency. Moreover, the left side of the arterial cone is not covered by large branches of the right coronary artery, and the section of the wall and its suture can be made without danger to the circulation.

Immediately on opening the pulmonary artery a large quantity of dark blood is expelled from the heart, and consequently the operating field must be narrowly walled off by the silk and cotton padded towels, in order to prevent the escape of the blood into the pleura.

The Performance of the Intracardiac Operation and the Closing of the Pulmonary Artery.—As soon as the incision is complete its edges are retracted by two mosquito forceps, the blood is sponged, and the valves are exposed. Air always enters the right ventricle. No special care is taken of this, since no complications due to the air emboli through the lungs were ever observed to follow. Three kinds of operation were performed: cauterization, suture and section, and suture of the valves. The cauterization of the valves was performed with the fine point of the thermocautery. The points of insertion of the valves, their free edge, or their internal surface were cauterized. The suture of the valves was made with a straight needle No. 16 and fine silk sterilized in vaselin. The left and right valves were united by one stitch (figure 3) at a distance of about two millimeters from the insertion to the arterial wall. This produced stenosis of the pulmonary orifice. Section and suture of one sigmoid valve were made in the following way: The sigmoid valves having been exposed by a long incision through the orifice, the right sigmoid was cut in its middle with the scissors, as far as the insertion to the arterial wall (figures 4 and 5). Afterwards the edges of the wound were united near the margin of the valve by a stitch made with straight needles No. 16 and silk sterilized in vaselin.

The operation completed, the pulmonary artery is sutured. In order not to lose any time a needle No. 12 with China silk No. 1 was kept ready. A minute piece of muscle had been fixed at the end of the thread, in order that no time should be spent in making a knot after the first stitch was made; the incision is closed with a continuous through and through suture. The time occupied by the incision of the pulmonary artery, the operation itself, and the suture of the artery varied between one minute and fifty seconds and three minutes.

Reëstablishment of the Circulation through the Heart.—As soon as the suture of the pulmonary artery is complete the clamp is removed. Generally during the period of the interruption of the circulation the heart is still beating feebly, but it may have stopped completely. As soon as the blood is allowed to flow from the vessel into the heart the pulsations recommence, weak at first, but very soon become quite normal. When the interruption of the circulation did not last more than two or two and one half minutes there was no need of massage; after a few minutes the heart had recovered its normal pulsations. A gauze sponge was always applied to the line of suture and a slight degree of compression made during a few minutes. When, after that time, there was still some leakage at the line of suture, one or two complementary stitches were added. Care was taken not to close the pericardium before the line of suture was absolutely without leakage. Then suture of the pericardium and of the thoracic wall is made, according to the ordinary method. The animal is dressed and taken care of as described in a previous article.[2]

EXPERIMENTAL.

The experiments were performed on ten medium sized dogs. Nine of these animals were young adult dogs in good health, and one was a dog about seven or eight years old. In three instances the operations consisted of exploration, suture, or section and suture of the sigmoid valves; in seven instances the sigmoid valves were cauterized.

Experiment 1. Exploration of the Sigmoid Valves of the Pulmonary Artery.—Black long haired dog. March 10, 1913. Etherization by the Meltzer-Auer method. Transverse left thoracotomy by the ordinary technique. Incision of the pericardium. Clamping of the pedicle of the heart with a soft-jawed forceps protected with rubber. Incision of the anterior wall of the pulmonary artery just above the sigmoid valves. The edges of the opening were retracted with two forceps. The sigmoid valves of the pulmonary artery could easily be seen and handled with the fingers or with the forceps. Then the incision of the pulmonary artery was closed by means of a continuous suture with China silk and a needle No. 12. The clamp was removed and the circulation reëstablished. The interruption lasted two minutes and fifty seconds. The pulsation of the heart started immediately without massage. After a few minutes it was normal. Closing of the pericardium and of the thoracic cavity by the ordinary method. The animal had no shock and walked about half an hour after the operation. During the afternoon it ate and drank as usual. May 20. Animal is still entirely normal.

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[2] Tuffier and Carrel, *loc. cit.*

Experiment 2. Suture of the Sigmoid Valves of the Pulmonary Artery.—Black and white long haired male setter dog. March 12, 1914. Etherization by the Meltzer-Auer method. Transverse left thoracotomy by the ordinary method and clamping of the pedicle of the heart. Section of the wall of the pulmonary artery and of the cardiac wall at the point of union of the anterior and left sigmoid valves. The valves were widely exposed by retraction of the edges of the wound by two forceps, and the right and left sigmoids were united at a distance of about 2 mm. from their insertion to the wall by a stitch made with a straight needle (Kirby No. 16). Then the edges of the incision of the pulmonary artery were sutured by the ordinary method. The clamp was removed after an interruption of the circulation lasting two minutes and twenty-five seconds. The pulsation of the heart started immediately without massage. Then the pericardium and the thoracic cavity were closed. After the operation the animal recovered, as in experiment 1. May 20, 1914. Animal is still in normal condition.

Experiment 3. Section and Suture of the Right Sigmoid Valve and of the Pulmonary Artery.—Yellow and white male fox-terrier, about seven or eight years old. March 17, 1914. Etherization by the Meltzer-Auer method. Opening of the chest by left transverse thoracotomy. Section of the pulmonary artery just above the pulmonary orifice, after the pedicle of the heart had been clamped. The sigmoid valves were exposed by retraction of the edges of the incision by two forceps. Then the scissors were introduced into the artery and the right sigmoid valve was completely sectioned in its middle. Next, the edges of the valve were approximated at the upper end of the incision by one stitch made with a needle No. 16 and fine silk sterilized in vaselin. The pulmonary artery was closed by the ordinary method and the circulation was reëstablished after an interruption of two minutes and thirty seconds. The operation was completed in the ordinary way and the animal not only sustained no shock but remained in excellent condition. May 20, 1914. Animal still is in excellent health. Slight diastolic murmur.

Experiment 4. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—Young brindle female bulldog. April 14, 1914. Etherization by the Meltzer-Auer method. Transverse thoracotomy and clamping of the pedicle of the heart by the ordinary method. Incision of the pulmonary artery just above the pulmonary orifice. The edges of the incision were retracted by two forceps and the sigmoid valves widely exposed. The blood was removed with sponges and the edges of the left and right sigmoid valves were cauterized with the thermocautery. Then the wall of the pulmonary artery was closed and the circulation reëstablished after an interruption of two minutes. The operation was completed by the ordinary method. The animal sustained no shock and remained in excellent condition, as in the preceding experiments. May 20, 1914. Animal is still in normal condition.

Experiment 5. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—Brindle female mongrel. April 20, 1914. Etherization by the Meltzer-Auer method. All the details of the operation were identical with those given in experiment 4. The interruption of the circulation lasted two minutes and five seconds. After the operation the animal remained in excellent condition. May 6, 1914. Animal is normal. May 8. Animal coughs. May 12. Died.

Autopsy.—Pneumonia, pericardial adhesions. Valves normal. Pulmonary incision healed, without deposit of fibrin.

Experiment 6. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—Brindle female mongrel. April 22, 1914. Etherization by the Meltzer-Auer method. Transverse thoracotomy and clamping of the pedicle of the heart by the ordinary method. Incision of the pulmonary artery just above the middle part of the anterior sigmoid valve. The incision was made as far as the base of the sigmoid sinus. Cauterization of the anterior sigmoid and of the point of insertion of the right and left sigmoids. Suture of the pulmonary artery and reëstablishment of the circulation after an interruption of two minutes and fifty seconds. Pulsation of the heart weak at first, normal after a few minutes. During the suture of the pericardium a small branch of the anterior coronary artery was wounded by the point of the needle. The hemorrhage was stopped by suture of the endocardium in front of the vessel. Hemorrhage ceased. The operation was completed in the ordinary way. The animal had no shock and in the afternoon was in excellent condition. April 28. Animal sick. April 29. Died.

Autopsy.—Wound infection; purulent pleurisy; no pericarditis, but pericardiac adhesions. Interior of heart normal; no thrombosis of pulmonary artery; thin layer of fibrin on the lower part of the line of suture; sigmoid valves normal; no deposit of fibrin at cauterized points (figure 6).

Experiment 7. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White and black mongrel. April 23, 1914. Etherization by the Meltzer-Auer method. The technique of the operation was identical with that used in experiment 6. But after the circulation had been interrupted and the pulsation of the heart had recommenced, fibrillary contractions suddenly appeared and the animal died. The fibrillary contractions were probably due to the fact that the incision had been made too low on the anterior part of the heart and that a deep stitch possibly included a branch of the coronary artery.

Experiment 8. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White female fox-terrier. April 28, 1914. Etherization by the Meltzer-Auer method. Incision of the skin at 10.10 A.M. Opening of the chest by transverse thoracotomy on the left side at 10.18. The pedicle of the heart was clamped at 10.30. The incision of the pulmonary artery, the cauterization of the valves, and the closing of the pulmonary artery were performed with the ordinary technique. The interruption of the circulation lasted one minute and fifty seconds. The operation was completed at 10.45. The animal was in excellent condition. May 20. Animal is still normal.

Experiment 9. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White and yellow male fox-terrier. April 29, 1914. Etherization by the Meltzer-

Auer method. Transverse thoracotomy. The heart was clamped for two minutes, during which time the incision of the pulmonary artery, cauterization of the valves, and suture of the arterial wall were made. The operation was completed and the dressing made. Same technique as in experiment 6. Animal was in excellent condition. May 20. Animal still normal.

Experiment 10. Cauterization of the Sigmoid Valves of the Pulmonary Artery.—White fox-terrier with brindle spots. April 30, 1914. Etherization by the Meltzer-Auer method. The technique was exactly the same as in experiment 9. At 11 A.M. incision of the skin. At 11.05 opening of the chest. At 11.09 opening of the pericardium. At 11.15 clamping of the heart, which lasted one minute and fifty seconds. During this period the pulmonary artery was cut, the valve cauterized, and the arterial wall sutured. At 11.27 the suture of the muscles was made, and at 11.32 the operation was completed. The animal was in normal condition. May 20. Animal is still normal.

RESULTS.

The results of these operations must be considered from the standpoint both of the general condition of the animals and of the modifications of the sigmoid valves of the pulmonary artery. Out of ten animals operated upon, three died and seven recovered and remained in excellent condition. The deaths were due to different causes. In experiment 5 the animal recovered completely and was apparently normal sixteen days after the operation. Then it sickened and died of pneumonia twenty days after the operation. In experiment 6 the cause of death which occurred seven days after the operation was a purulent pleurisy. In experiment 7 the animal died on the operating table of fibrillary contractions of the heart. It is probable that some of the causes of death can be eliminated. The infection of the thoracic wall and the purulent pleurisy, as well as the pneumonia which occurred in experiment 5, are preventable complications. They were caused by faulty technique which can be avoided. In all instances in which a few persons only were present in the operating room, the operation could be conducted with great care and all the animals recovered. It is probable that the proportion of deaths obtained in the first series of experiments will be much lower in the future. The general condition of the animals that survived was excellent. They sustained no shock; usually one hour after the operation they walked about, and three or four hours afterwards they ate and drank. These results were observed not only on young dogs, but also on a dog seven or eight years old. All the animals were operated upon more than one month ago, and are at present normal.

The local modifications brought about by the operations were studied on the two specimens taken from animals 5 and 6, and also from the clinical examination of the seven surviving animals. The heart taken from animal 6 showed the result of the cauterization of the sigmoid valves of the pulmonary artery seven days after the operation. There were pericardiac adhesions on the anterior wall of the heart, but the heart itself was apparently normal. No thrombosis of the pulmonary artery existed. Above the anterior valve was the cicatrice of an incision one and one half centimeters long, which extended almost to the bottom of the sigmoid sinus (figure 6). The lower part of the incision was covered by a thin film of fibrin. In the upper part the stitches could be seen; the union of the edges of the wound was perfect. The margin of the anterior valve was irregular, owing probably to the fact that it had been cauterized. But there was no apparent deposit of fibrin. The left and right valves were normal. In experiment 5 the specimen showed the result of the operation after twenty-two days. The anterior and posterior parts of the heart were almost completely adherent to the pericardium. The cicatrice of the incision was seen just above the anterior valve near its left insertion in the anterior wall. It was entirely cicatrized, the stitches could not be seen, being covered by a smooth, glistening membrane. The margin of the right sigmoid was slightly irregular, owing probably to the cauterization. The two other sigmoids were normal. The examination of the two specimens demonstrated that it was possible to make a section of the wall of the pulmonary artery without injuring the valves, and that the cauterization of the valves does not produce thrombosis.

The condition of the heart of the seven animals that remained in good health was examined clinically. Six of the animals were entirely normal. In experiment 3 the animal presented a slight diastolic murmur. This animal had undergone a section of the right sigmoid valve and suture of the valve by one stitch placed near the margin. It is probable that the bottom of the valve did not unite and that a slight amount of insufficiency persisted.

CONCLUSION.

Incision, suture, and cauterization of the sigmoid valves of the pulmonary artery have been performed successfully in dogs. In the first series of ten animals, there were only three accidents, probably from largely preventable causes, leading to the death of the animals.

EXPLANATION OF PLATES.

PLATE 3.

FIG. 1. Incision of the pulmonary artery at the union of the anterior and left sigmoid valves.

PLATE 4.

FIG. 2. Same as figure 1.

FIG. 3. Suture of the right and left sigmoid valves.

FIGS. 4 and 5. Section and suture of the right sigmoid valve.

PLATE 5.

FIG. 6. Specimen taken seven days after the cauterization of the sigmoid valves.

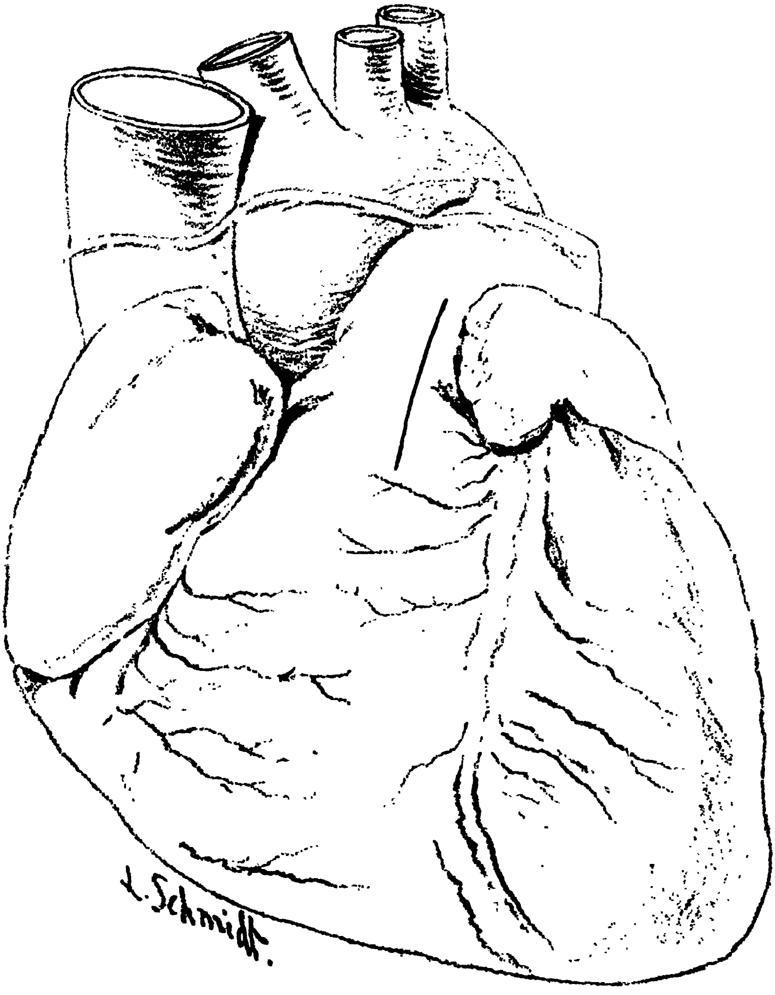

FIG. 1.

(Carrel: Operations on Sigmoid Valves of Pulmonary Artery.)

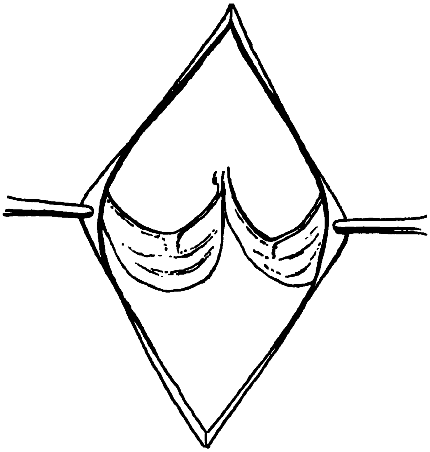

FIG. 2.

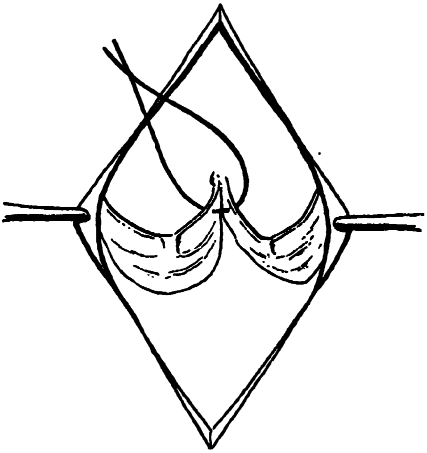

FIG. 3.

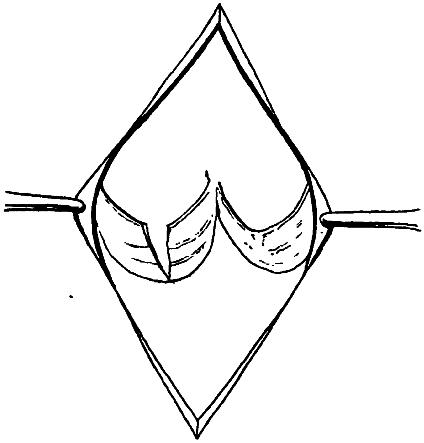

FIG. 4.

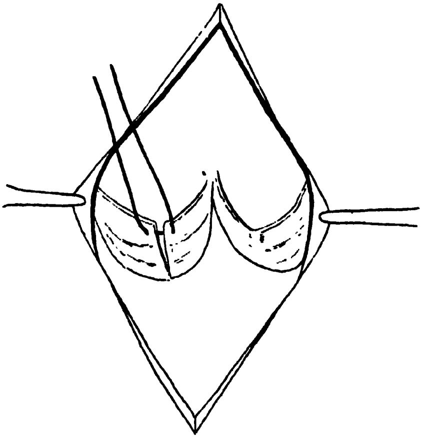

FIG. 5.

(Carrel: Operations on Sigmoid Valves of Pulmonary Artery.)

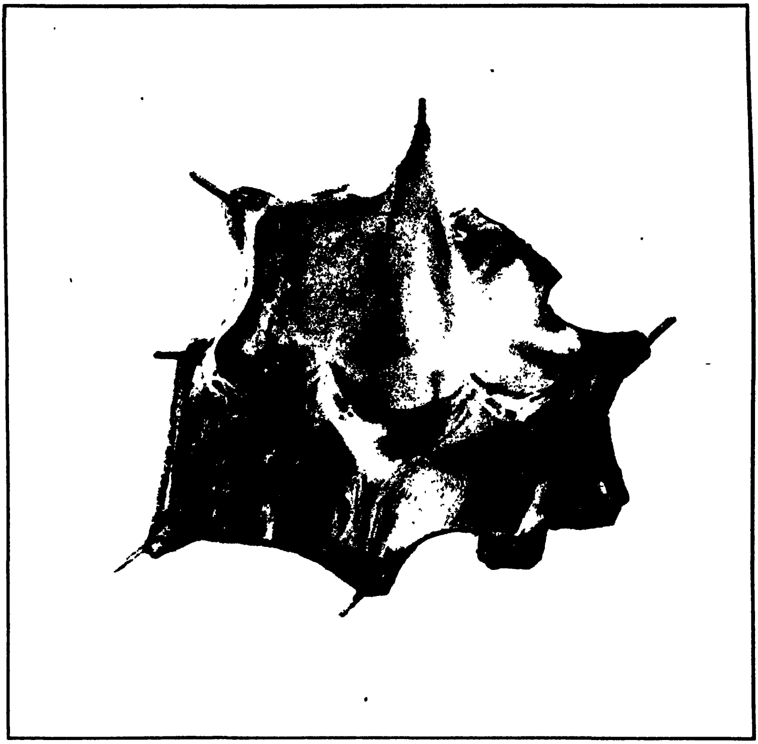

FIG. 6.

(Carrel: Operations on Sigmoid Valves of Pulmonary Artery.)

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, August 1, 1914, Vol. xx, No. 2, pp. 130-139.]

THE EFFECT OF THE VARIATION IN THE OSMOTIC TENSION AND OF THE DILUTION OF CULTURE MEDIA ON THE CELL PROLIFERATION OF CONNECTIVE TISSUE.*

BY ALBERT H. EBELING.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 11 TO 14.

In his fundamental experiments Jacques Loeb has shown the importance of the osmotic tension of water for the growth of marine organisms. Carrel and Burrows,[1] by cultivating for a few days embryonic chick spleen in diluted plasma and in plasma the osmotic tension of which had been modified, attempted to determine what factors could activate the rate of growth of the tissues of higher animals. These experiments were made before Carrel developed the technique which rendered possible the permanent life of connective tissue *in vitro*. Afterwards it became apparent that the modifications of the medium acted differently on tissues proliferating for many generations and tissues maintained for a few days in a condition of survival, and that the conclusions in the article of Carrel and Burrows needed to be modified.

Dr. Carrel gained the impression that it would be necessary, in order to know the influences on cell proliferation of the variation in the osmotic tension and of the dilution of the culture media, to observe for many passages the growth of a tissue in a specific medium. The present experiments were therefore undertaken to determine definitely to what extent strains of connective tissue, kept in a condition of active life, *in vitro,* for many generations, were influenced by the modification of the medium. During the time this work was in progress, an article by Lambert[2] was published on the effect of dilution of plasma on the growth of cells in tissue

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* Received for publication, May 25, 1914.

[1] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.,* 1911, xiii, 562.

[2] Lambert, R. A., *Jour. Exper. Med.,* 1914, xix, 398.

cultures. It was stated that plasma with isotonic solutions causes a more extensive migration in cultures of cells of the actively migratory type, such as those of spleen and bone marrow. Dilution with a limited quantity of distilled water produced the same effect. Less actively motile cells are influenced little or not at all by dilution; also, dilution of the plasma with either isotonic solutions or distilled water is without effect on the cell multiplication. His experiments did not differ in method from those of Carrel and Burrows and are, therefore, open to the same objection.

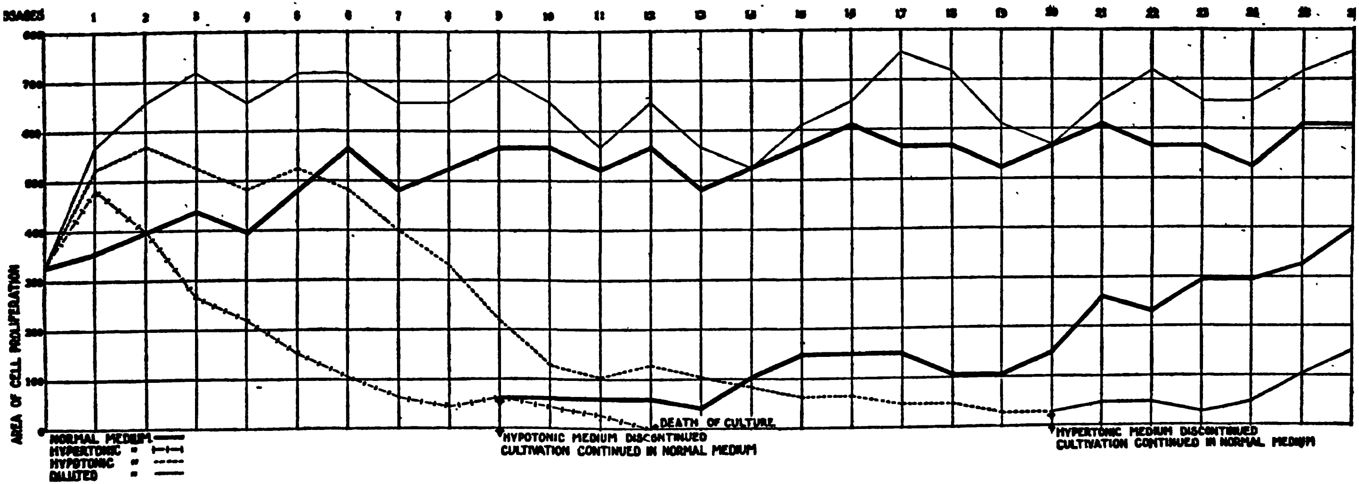
TECHNIQUE.

The strain of connective tissue used as a control was cultivated in a medium, designated normal, composed of one part of freshly prepared embryonic chick extract and of two parts of normal chicken plasma.

Hypotonic medium was prepared by adding two parts of distilled water to three parts of normal plasma and one part of fresh embryonic chick extract.

Preliminary experiments were made to determine the concentration of the salt solution which was added to normal plasma in order to render it sufficiently hypertonic. It was found that a 2.5 per cent. sodium chloride solution was too concentrated. When employed in a culture medium it proved distinctly unfavorable to cell proliferation after one or two passages. A medium containing a 2 per cent. solution of sodium chloride in the same proportion showed that it was possible to keep strains of connective tissue cells in a condition of active life for a number of passages. 1 and 1.5 per cent. solutions were also tried, but the hypertonicity produced thereby was found to be so slight as to approach the conditions which prevailed when normal plasma was diluted with Ringer solution. The hypertonic medium was prepared by adding two parts of a 2 per cent. solution of sodium chloride to three parts of normal chicken plasma and one part of fresh embryonic chick extract.

Diluted medium was prepared by adding two parts of Ringer solution to three parts of normal chicken plasma and one part of fresh embryonic chick extract. Thus, the three modified media, hypotonic, hypertonic, and diluted, contained the same relative amount of normal plasma and fresh embryonic chick extract.



TEXT-FIG. 1. The average area of cell proliferation which developed during forty-eight-hour intervals (one passage) of incubation at 40° C. in cultures of connective tissue which were cultivated in normal, hypertonic, hypotonic, and diluted media, respectively.

Two strains of connective tissue cells were used in these experiments. One strain was derived from a fragment of heart tissue from a chick embryo seven days old, extirpated on January 17, 1912; the other from a fragment of heart tissue from a chick embryo eight days old, isolated on February 18, 1913.

Cultures were made by subdividing fragments of these two strains of connective tissue. The pieces were washed in Ringer solution for three quarters of a minute to one minute and cultivated respectively in normal, hypotonic, hypertonic, and diluted medium. After forty-eight hours' incubation the cultures were washed in Ringer solution as before, transferred into fresh medium, and again incubated. After incubation for one, six, twenty-four, and forty-eight hours, observations were made, and in some instances cultures were fixed and stained. To determine the influence of modified media on the proliferation of connective tissue cells, the growth obtained in a culture, after stated periods in normal medium, was taken as a standard of comparison. The extent of this growth was measured with an ocular micrometer and recorded. The accompanying chart shows, in curves, the relative area of cell proliferation which developed during a passage (forty-eight hours) in cultures which had been grown in modified media. Calculations were made by measuring in the different media the width of the zone of new growth which had developed during a passage. The measurements for all cultures which had been cultivated in the same medium were averaged and the area was computed. The relative density of cell proliferation was determined by observations on stained preparations, and photomicrographs of the preparations were made.

EXPERIMENTS.

Continued cultivation of connective tissue in hypertonic medium produced the following results: When a culture was transferred to hypertonic medium from a normal medium in which the width of the area of cell proliferation after forty-eight hours' incubation was equal to seven divisions of the ocular micrometer, observations showed, after one hour's incubation, no evidence of cell proliferation; after six hours' incubation a few elongated cells were observed spreading from the periphery of the central fragment into the medium; after twenty-four hours the area of cell proliferation measured, on the average, six and a half divisions, slightly more than the area of cell proliferation in the control, which measured six divisions. Stained preparations at this stage of incubation showed the area to be denser than the control. After forty-eight hours' incubation the zone of proliferating cells measured nine divisions, and in the control seven and a half divisions. The density was greater in comparison with the normal control and the contrast could be observed in the living culture. Examination of the cells in these cultures showed the presence of many refractile globules of varying size in the cytoplasm of the cell. In the control, cells showed small refractile globules, few in number and fairly uniform in size. Preparations stained with Sudan III and hematoxylin showed these refractile globules to be fatty substances which had accumulated in the cell cytoplasm. A third passage into hypertonic medium after forty-eight hours' incubation produced a less extensive, though quite as dense an area of cell proliferation as the control. The zone measured six divisions, and the control nine and a half. The amount of fat present in the cytoplasm of the cell was observed to be apparently as great as in the previous passage. There was no increased amount of fat present in the control over that present in the new cells which developed in the previous passage. A fourth and fifth passage, after forty-eight hours' incubation, respectively, showed a decided decrease in extent of new growth. The area of cell proliferation appeared to be as dense as the area of cell proliferation in the control, but the central portion of the culture had become a thick and opaque mass. The cells were observed to be filled with large fat granules. After the sixth and seventh passages the extent of cell proliferation was decidedly less than the control, measuring two and a half divisions, whereas the extent of growth in the control measured nine and a half. The central portion of the cultures had contracted into a very dense, spherical, opaque mass. Further passages into hypertonic medium resulted in a rapid degeneration of the culture, with central necrosis and death after the eleventh or twelfth passage. In some instances tissues which had been cultivated for nine passages (eighteen days) in hypertonic medium were revived by again cultivating them in normal medium, and after seventeen passages (thirty-four days) the extent of cell proliferation was approaching normal. In the first few days during which a culture was cultivated in hypertonic medium the area of new growth was more extensive and apparently more dense than in the control. After that time this area decreased rapidly and eventually the culture died.

Cultivation of connective tissue in hypotonic plasma after one hour's incubation showed new cells budding out from the peripheral portions of the central fragment. The same condition was observed in the control. After six hours' incubation the area of cell proliferation was about the same as the control, but in twenty-four hours the zone of proliferating cells was more extensive than in the control, measuring about eight and a half divisions in width, whereas the width of the area in the control measured slightly over seven divisions. After forty-eight hours' incubation the increase in extent of cell proliferation was decidedly greater than the control, measuring about nine and a half divisions, as compared with seven and a half divisions in the control. The density of growth, however, was less than the control. Stained preparations showed fewer planes in which cell proliferation had taken place. After the second and third passages, respectively, the extent of growth was still observed to be greater (about nine and a half divisions) than the control (about seven and a half divisions). The accumulation of fat globules in the cytoplasm of the cells was practically the same as the control. After the fourth and fifth passages the area of proliferating cells was slightly greater than the control, but the relative density was less in the former. After the sixth and seventh passages the extent of cell proliferation decreased and was observed to be less than the control. The cells showed an increase in fat accumulation over the control and the cells appeared loosely joined, forming a large meshed network of interlacing cells. Continued cultivation in hypotonic medium up to twenty passages (forty days) showed a gradual decrease in the extent of growth. The central fragment became dense and opaque and large fat globules were observed in the cytoplasm of the cells. After twenty passages in hypotonic medium some cultures were again cultivated in normal medium. They usually recovered after five or six passages. During a period lasting about ten days, a culture cultivated in hypotonic medium showed that the area of cell proliferation was more extensive though less dense than the normal control. After that time the extent of growth decreased gradually and finally cell proliferation became sluggish. It was possible to revive again a culture at this stage.

The cultivation of cultures of connective tissue in diluted medium after one hour's incubation showed a number of new cells growing out into the medium from the periphery of the central fragment. In six hours an appreciable crown of new cells encircled the central portion of tissue, and after twenty-four hours the area of new growth was more extensive (about eight and a half divisions in width) than the control (about seven divisions in width). After forty-eight hours it was observed that the area of cell proliferation which had developed was decidedly more extensive (width of area eleven divisions) than the control (width of area seven and a half). Stained preparations showed this area to be less dense than the control, but there was no increase in the amount of fat globules present in the cytoplasm of the cells. A second, third, and fourth passage showed an increase in area over the control and many passages thereafter (twenty-two passages, forty-four days) showed that this increase in area of cell proliferation over the normal was fairly constant. This point is shown in the text-figure 1. The fluctuations of growth which were observed in the control were also observed at the same time in the experiment. When the extent of cell proliferation was less extensive in normal medium than in the previous passage, it was found that there was a relative decrease, on most occasions, in the extent of cell proliferation in diluted medium. The stained preparations of cultures cultivated in diluted medium for many passages showed an extensive, loosely meshed network of elongated cells. The control showed a more densely packed mass of elongated cells, but the area of cell proliferation was less. Figure 1 represents an entire culture of connective tissue forty-eight hours after passage into normal medium, which had been cultivated for about two hundred passages in this medium. Figure 2 shows an entire culture of connective tissue forty-eight hours after the last passage into diluted medium. The last passage was the twenty-fifth consecutive passage into diluted medium. Figure

3 is a higher magnification of part of a control culture after forty-eight hours' incubation. Figure 4 shows a portion of the area of cell proliferation which developed in a culture in forty-eight hours in diluted medium, after twenty-two passages into the same medium. The relative increase in the extent of cell proliferation in diluted medium, over the control, remained fairly constant through the total number of passages, although no actual increase in mass was observed (text-figure 1).

When connective tissue was cultivated in hypertonic medium for one passage and then in hypotonic medium, observations showed no marked differences in cell proliferation, but alternate passages from hypertonic to hypotonic medium for three or four passages caused death of the culture. Alternate passages from hypertonic medium into diluted medium proved unfavorable after three or four passages, and usually resulted in death of the culture after five generations. Alternate passages from hypertonic medium into normal medium proved unfavorable after five passages, and when continued for ten passages the culture died. Alternate passages from hypotonic into normal medium were continued for ten generations. A gradual decrease in the extent and activity of cell proliferation was observed and this treatment proved unfavorable for growth in the culture. Observations showed that alternate passages from normal medium into diluted medium did not appear to affect the dynamic condition of the culture, although, relatively, there appeared to be a denser area of cell proliferation in normal medium and a less dense but more extensive area of growth in diluted medium. Alternate passages of connective tissue into modified media in most instances produced unfavorable results.

SUMMARY.

For the first few days of cultivation of connective tissue in hypertonic, hypotonic, and diluted medium, cell proliferation was stimulated. The first outgrowths of new cells in the modified media did not occur sooner than in normal medium. In hypertonic medium the density of the area of cell proliferation appeared to be greater than the control, but in hypotonic or diluted medium there seemed to be no increase in actual mass over the control. These observa-

tions confirm the conclusions of Carrel and Burrows, as well as those of Lambert.

Subsequent to the first few days of cultivation in hypertonic medium the area of cell proliferation decreased and in a short time conditions developed which were unfavorable to growth, and finally resulted in death of the culture, unless it revived before this stage. Hypotonic medium after about ten days no longer caused more extensive areas of proliferating cells; but instead, the extent of new growth gradually decreased, and the culture merely remained alive unless revived. In diluted medium the extent of the area of cell proliferation remained greater with no actual increase in mass. The area of cell proliferation which is observed during the first few days in a culture of fresh tissue recently extirpated does not indicate the actual influence of modified media. It was only after continued cultivation of strains of connective tissue in these modified media that their influence on cell proliferation was determined.

CONCLUSION.

An increase or decrease in the osmotic tension of the culture medium at first stimulates cell proliferation, but eventually retards it and proves to be unfavorable to growth. Dilution of the medium without change of the osmotic tension produces a more extensive zone of cell proliferation but no increase in the actual mass of newly formed tissue. A culture of connective tissue, which has been growing under unfavorable conditions, due to changing the osmotic tension of the medium in which it has been cultivated, is capable of being revived.

EXPLANATION OF PLATES.

Plate 11.

Fig. 1. The extent of growth obtained after forty-eight hours' incubation in normal medium. This culture had been cultivated in normal medium for about two hundred passages. Control for culture in figure 2. Stained preparation; low power.

Plate 12.

Fig. 2. The extent of growth obtained after forty-eight hours' incubation in diluted medium. This culture had been cultivated in diluted medium for twenty-five passages (fifty days). Stained preparation; same magnification as figure 1.

PLATE 13.

FIG. 3. The extent of the area of cell proliferation which developed after forty-eight hours' incubation in a normal control. Stained preparation; higher power.

PLATE 14.

FIG. 4. The area of cell proliferation which developed after forty-eight hours' incubation in diluted plasma. This culture had been cultivated in the same medium for twenty-three generations (forty-six days). Stained preparation; same magnification as figure 3.

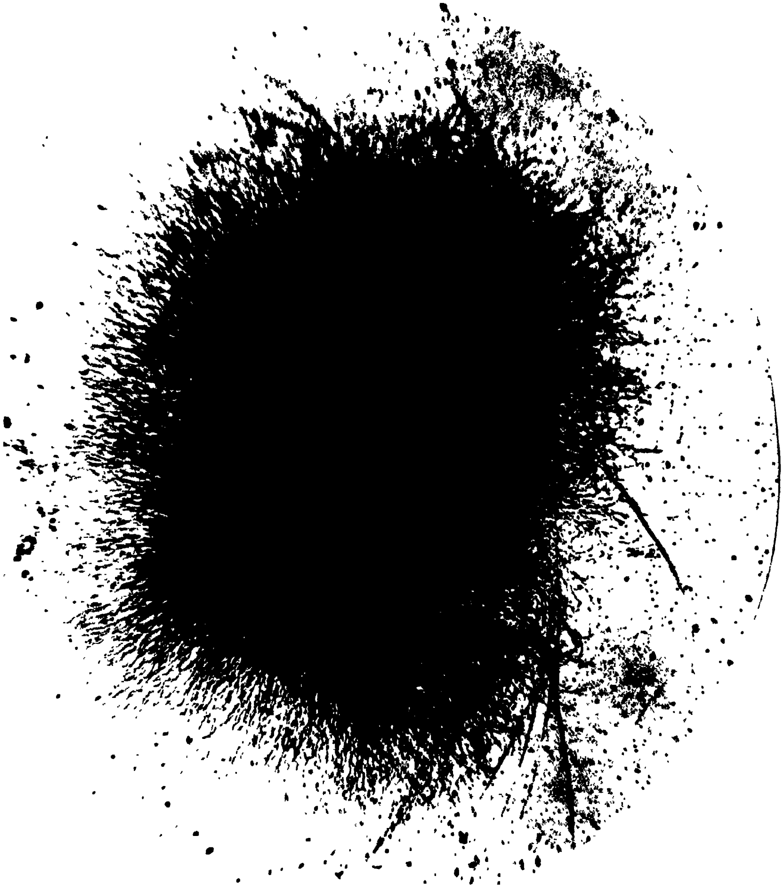


FIG. 1.

(Ebeling: Cell Proliferation of Connective Tissue)

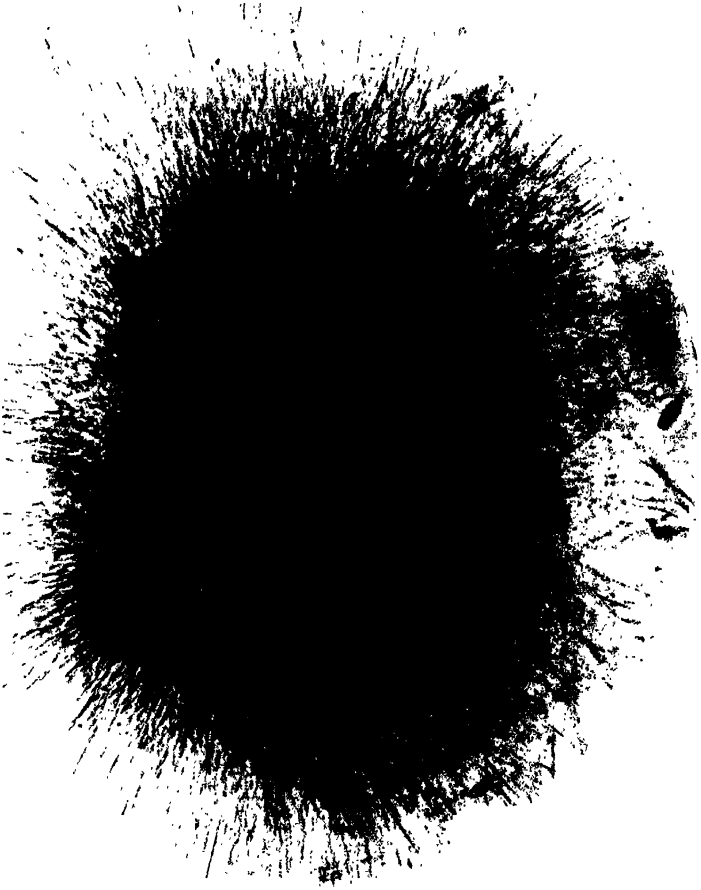


FIG. 2.

(Ebeling: Cell Proliferation of Connective Tissue.)

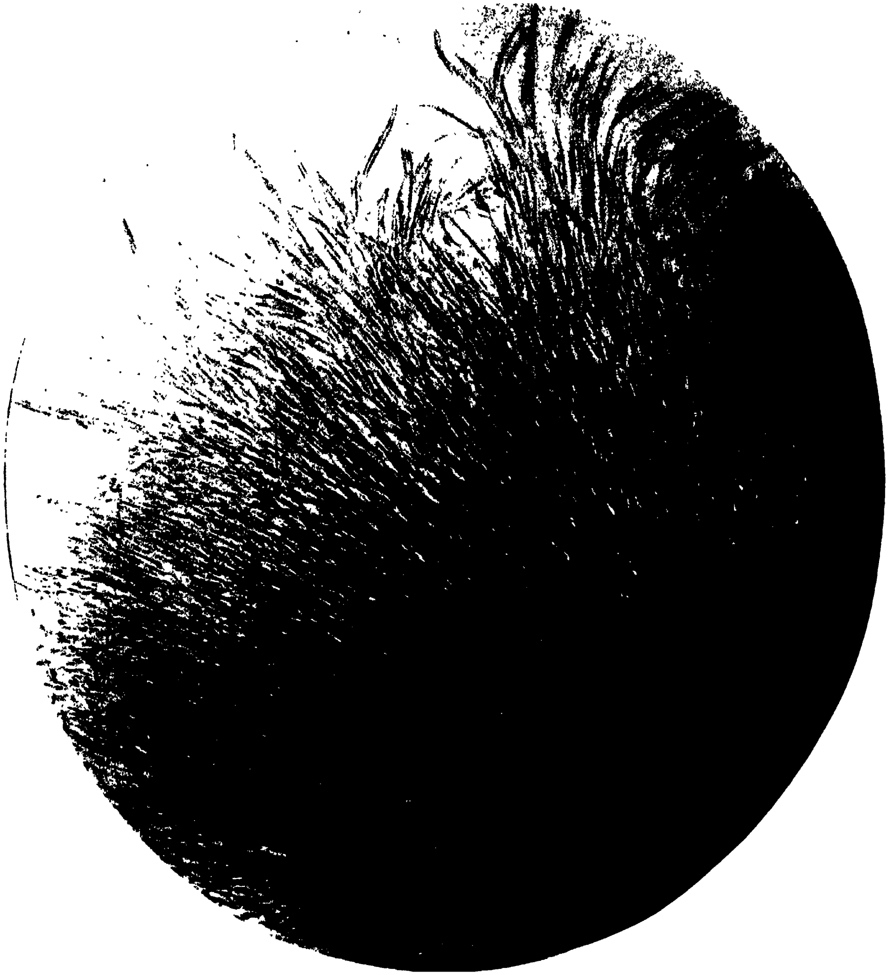


FIG. 3.

(Ebeling: Cell Proliferation of Connective Tissue.)

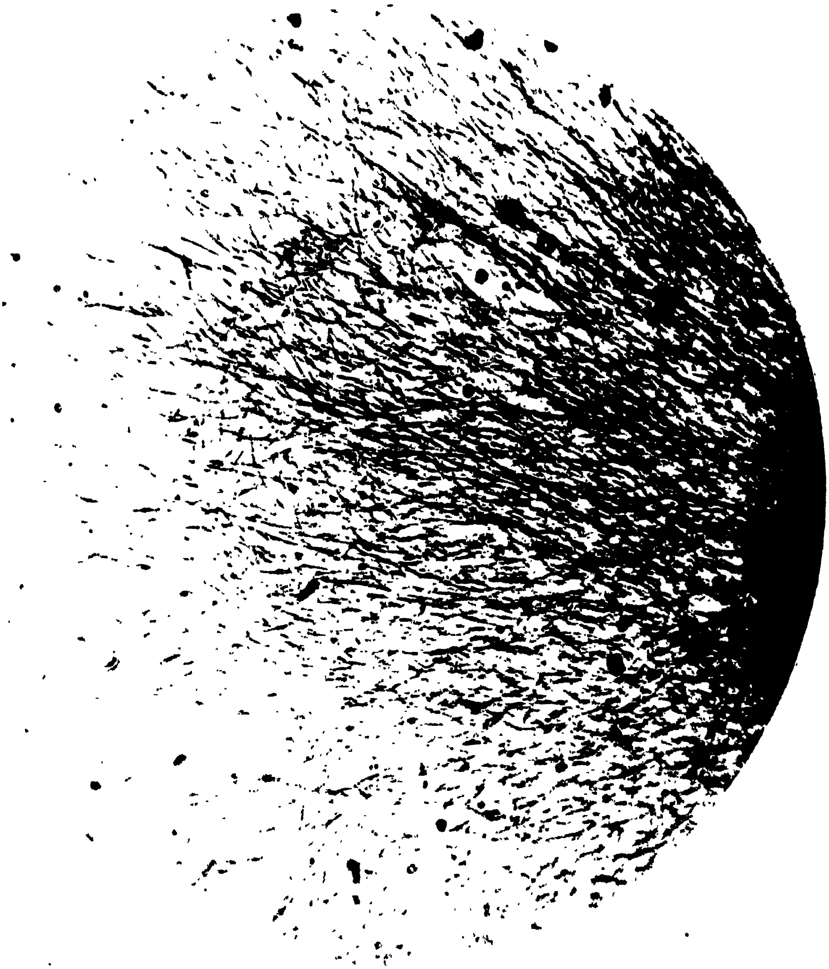


FIG. 4.

(Ebeling: Cell Proliferation of Connective Tissue.)

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THE CULTIVATION OF HUMAN SARCOMATOUS TISSUE IN VITRO.*

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PLATES 15 TO 17.

The first attempt to cultivate human malignant tumor *in vitro* was made in 1911 by Carrel and Burrows.[1] Small fragments of tumor were cultivated in normal human plasma and incubated. It was observed in some cases that after a few days the fragments were surrounded by many cells; but generally liquefaction of the medium occurred. The tissues were kept in a condition of survival for a few days, but no real cultures were obtained.

Lately it became possible to keep human fetal tissue, derived from fresh cadavers, in a condition of independent life for several generations,[2] and we therefore attempted to cultivate human sarcomatous tissue in the same manner.

TECHNIQUE.

The medium employed in these experiments was composed of equal parts of normal human plasma and Ringer solution and varying quantities of extract.

The extract was prepared by cutting tissues obtained from fresh fetal cadavers into small pieces, and adding an equal quantity of Ringer solution. After forty-eight hours in cold storage the substance was centrifuged and the supernatant fluid pipetted off. This fluid was used as extract in the making of cultures.

The tissues employed were obtained from recently excised sarcomatous growths,[3] and cultures were made about one and a half hours

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* Received for publication, June 3, 1914.

[1] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 387.

[2] Losee, J. R., and Ebeling, A. H., *Jour. Exper. Med.*, 1914, xix, 593.

[3] The sarcomatous growths were obtained by Dr. Carrel, through the courtesy of Dr. W. B. Coley, from some of his cases at the General Memorial Hospital, New York. Immediately after excision the tissues were carried to the Laboratories of the New York Lying-In Hospital.

after excision. The primitive cultures were made by putting small, thin fragments of this tissue into the medium. After coagulation the cultures were immediately placed in the incubator and incubated at 38° C. for 24, 48, and 72 hours, the time of passage into fresh medium being governed by conditions which developed in the culture. Before the fragments in cultures were transferred into fresh medium they were washed in Ringer solution for about one minute.

EXPERIMENTS.

Experiment 1, Series 1, Cultures 1, 2, 3, and 4.—Fragments from the periphery of an osteosarcoma[4] were cultivated in equal parts of human plasma and Ringer solution, to which one fourth part of extract was added. The cultures were made about one hour and a half after excision of the growth and incubated.

After 1, 6, and 18 hours' incubation there was no evidence of cell proliferation in any of the cultures. In twenty-four hours there was still no growth to be observed and the medium had become liquefied around the fragments. The cultures were therefore washed and changed into fresh medium (first passage), to which one half part of extract was added. After twenty-four hours' incubation, growth was found to be present in culture 3, but no growth was observed in cultures 1, 2, and 4, and in forty-eight hours there was still no evidence of cell proliferation in these cultures; the medium had become liquefied, and they were therefore discarded.

Culture 3 was cultivated in the same medium (second passage), and in forty-eight hours growth was apparent. This culture was transferred into fresh medium for twelve passages, during which time (twenty-one days) growth was observed after each transfer into fresh medium. The culture was stained and photographed (figure 1).

Series 2, Cultures 1, 2, 3, and 4.—Fragments from the peripheral area of the same growth were cultivated in the same manner as series 1.

After 1, 6, and 18 hours' incubation there was no evidence of cell proliferation. The medium was still in good condition. In twenty-four hours no growth was observed, and the medium about the

[4] Pathological diagnosis: large round cell sarcoma.

pieces in cultures 1, 2, and 3 had become liquefied. Culture 4 was allowed to remain in the incubator. It was examined after forty-eight and seventy-two hours, but there was no evidence of cell proliferation, although the medium was still in good condition. The culture was discarded. After twenty-four hours cultures 1, 2, and 3 were changed into fresh medium (first passage), to which one half part of extract was added.

Culture 1 after twenty-four hours showed no growth, and the medium was completely liquefied. It was changed into fresh medium (second passage), the same proportion of extract being added as in the previous passage. In twenty-four hours a few scattered cells were observed, but after forty-eight and seventy-two hours there was no further increase in the extent of cell proliferation. The culture was discarded.

Culture 2 (first passage) after twenty-four hours' incubation showed an area of cell proliferation, with no liquefaction of the medium. In seventy-two hours a good growth had developed. The medium was slightly liquefied. It was then changed into fresh medium (second passage), to which one fourth part of extract was added. After twenty-four hours growth had developed, and in forty-eight hours the area of cell proliferation was more extensive, but liquefaction of the medium had developed. The culture was changed into fresh medium (third passage) with the same proportion of extract added as in the previous passage. After twenty-four hours a few cells were observed in the medium surrounding the central fragment. In forty-eight hours there was no increase in the extent of growth and the medium had liquefied. The culture was again transferred into fresh medium (fourth passage), one part of extract being added. In twenty-four hours the medium had become liquefied and no growth was observed. The culture was changed (fifth passage) into fresh medium, the same proportion of extract being added as in the previous passage. In twenty-four hours growth had developed, and in forty-eight hours it was more extensive, but the medium was almost completely liquefied. The culture was transferred (sixth passage) into fresh medium in the same manner as in the previous passage. After twenty-four hours cell proliferation was observed, but small colonies of bacteria had also developed. The infection was general and the culture was discarded.

Culture 3 (first passage), after twenty-four hours' incubation, showed a few scattered cells which had grown out from the original fragment. In forty-eight hours the growth of new cells had increased, but the medium was slightly liquefied. The culture was then treated (second passage) in the same manner as in the previous passage. After twenty-four hours good growth had developed and the medium was in good condition. After forty-eight hours the growth was more extensive, but after seventy-two hours there was no further increase and the medium was slightly liquefied. The third passage into fresh medium was then made, the proportion of extract being increased to one part. After twenty-four hours' incubation the growth had developed and the medium was in good condition. In forty-eight hours the culture was growing actively and the medium had become slightly liquefied. The culture was subsequently changed into fresh medium for twenty-one more passages. The extent of growth fluctuated and gradually decreased. In the fifteenth passage the culture was divided and two cultures were made; one of the cultures (figure 2) was fixed after forty-eight hours' incubation. After the twenty-fourth passage cell proliferation stopped. This culture was transferred twenty-five times, during a period of fifty-one days. The entire history is given in table I, which shows the passage, treatment, and observations that were made on culture 3.

Experiment 2.—A series of cultures, Nos. 1, 2, 3, and 4, was made from fragments of the periphery of a large round cell sarcoma,[5] and cultivated in equal parts of plasma and Ringer solution. After twenty-four hours there was no evidence of growth in any of the cultures and the medium was in good condition. In forty-eight hours there was evidence of cell proliferation in all cultures. After seventy-two hours the area of cell proliferation had increased, but the medium in cultures 3 and 4 had become liquefied. Cultures 1 and 2 were stained.

Cultures 3 and 4 were changed into fresh medium (first passage), and one part of extract was added to the medium. Both cultures developed good growth in forty-eight hours with no liquefaction of the medium. They were again transferred into fresh medium

[5] **Pathological diagnosis: large round cell sarcoma.**

TABLE I.

Passage.	Date (1913).	Treatment of culture.	Observations.
Experiment 206–3	Nov. 18	Culture of a fragment of the peripheral part of an osteosarcoma, cultivated in 1 part of human plasma, 1 part of Ringer solution, and ½ part of extract	Nov. 19. Medium liquefied; no growth.
1	Nov. 19	Washed in Ringer solution for 1 minute, cultivated in 1 part of human plasma, 1 part of Ringer solution, and ½ part of extract	Nov. 20. A few scattered cells; medium in good condition. Nov. 21. Good growth; medium slightly liquefied.
2	Nov. 21	Treated in the same manner as in previous passage	Nov. 22. Good growth; medium in good condition. Nov. 23. Growth more extensive; medium in good condition. Nov. 24. No increase in extent of growth; medium slightly liquefied.
3	Nov. 24	Washed in Ringer solution for 1 minute, cultivated in 1 part of human plasma, 1 part of Ringer solution, and 1 part of extract	Nov. 25. Good growth; medium in good condition. Nov. 26. Growing actively; medium slightly liquefied.
4	Nov. 26	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 27. Good growth; medium in good condition. Nov. 28. Growth more extensive; medium in good condition. Nov. 29. No increase in extent of growth; medium liquefied.
5	Nov. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 30. A few cells; medium in good condition. Dec. 1. A few cells; medium slightly liquefied.
6	Dec. 1	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 2. A few scattered cells; medium in good condition. Dec. 3. Good growth; medium in good condition.
7	Dec. 3	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 4. Good growth; medium in good condition. Dec. 5. Very good growth; medium in good condition.
8	Dec. 5	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 6. Good growth; medium slightly liquefied.
9	Dec. 6	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 7. Good growth; medium in good condition. Dec. 8. More extensive growth; medium liquefied.
10	Dec. 8	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 9. Good growth; medium partially liquefied. Dec. 10. No increase in amount of growth; medium liquefied.

TABLE I.—*Continued.*

Passage.	Date (1913).	Treatment of culture.	Observations.
11	Dec. 10	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 11. Good growth; medium in good condition. Dec. 12. Growth increased; medium in good condition.
12	Dec. 12	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 14. Good growth; medium in good condition. Dec. 15. Very good growth; medium in good condition.
13	Dec. 15	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 16. Growing; medium in good condition. Dec. 17. Very good growth; medium slightly liquefied.
14	Dec. 17	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 18. Good growth; medium in good condition. Dec. 19. Extensive growth; medium in good condition.
15	Dec. 19	Washed in Ringer solution for 1 minute, divided into 2 parts, and cultivated in the same medium (cultures 1 and 2)	Dec. 20. Good growth in cultures 1 and 2; medium in good condition. Dec. 21. Good growth; medium in good condition, No. 2 fixed and photographed. Dec. 22. Good growth; medium in good condition.
16	Dec. 22	Washed in Ringer solution for 1 minute, divided into 2 parts, and cultivated in the same medium	Dec. 23. Growing; medium in good condition. Dec. 24. Growing; medium partially liquefied.
17	Dec. 24	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 26. Growing; medium in good condition.
18	Dec. 26	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 27. A few cells; medium in good condition. Dec. 28. Growing; medium in good condition. Dec. 29. Growing; medium in good condition.
19	Dec. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 30. A few cells; medium in good condition. Dec. 31. Growing; medium in good condition.
20	Dec. 31	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 2. Growing; medium in good condition.
21	(1914) Jan. 2	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 3. No growth; medium in good condition. Jan. 4. A few cells.
22	Jan. 4	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 5. A few cells; medium in good condition. Jan. 6. Slow growth; medium slightly liquefied.
23	Jan. 6	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 7. No growth. Jan. 8. A few scattered cells; medium in good condition. Jan. 9 Growing slowly; medium slightly liquefied.

TABLE I.—*Concluded.*

Passage.	Date (1914).	Treatment of culture.	Observations.
24	Jan. 9	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 10. Slight growth; medium in good condition. Jan. 11. No increase in growth; medium in good condition.
25	Jan. 11	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 12. No growth. Jan. 13. No growth. Jan. 14. Discarded.

(second passage). Culture 4 showed a few proliferating cells after seventy-two hours' incubation, and the medium was in good condition. The culture was discarded. After twenty-four hours' incubation in the second passage culture 3 showed growth with no liquefaction. In forty-eight and seventy-two hours growth had increased, but the medium was partially liquefied. The culture was transferred into fresh medium (third passage) and showed a few cells which had spread out into the medium from the central fragment. After seventy-two hours there was no increase in the number of cells, and the medium had liquefied. The culture was transferred into fresh medium (fourth passage), and after forty-eight hours good growth was observed. The medium was in good condition. The fifth passage into fresh medium was made in the same manner as in the previous passage. After twenty-four hours the medium was totally liquefied and no growth was observed. The fragment was transferred to fresh medium, but after 24, 48, and 72 hours no growth developed. The culture was discarded.

RESULTS.

Two experiments were made in which fragments from human sarcomatous tissue were cultivated. It was possible to keep cultures of such tissue in a condition of active life *in vitro* for several generations.

During the first twenty-four hours of incubation there was usually no evidence of cell proliferation, and slight liquefaction around the primitive fragments. When no liquefaction occurred, growth of new cells manifested itself after forty-eight hours. Twenty-four hours after passage into fresh medium (first passage), cell proliferation was observed in those cultures which showed no evidence of growth when first cultivated. In comparison with human connective tissue, the rate of growth was practically the same in the beginning, but a gradual decrease in the activity and extent of cell proliferation was observed as the length of time increased during which the culture was carried through successive passages. Microscopic examination of the first outgrowth of cells showed the presence of large, round, as well as elongated and ramified cells. In subsequent passages the round cells were no longer to be identified, and the elongated, ramified variety only were observed. The morphological characteristics of these cells did not appear to differ from the cells present in cultures of normal human connective tissue. Preparations stained with Giemsa stain showed the large round cells as having a densely stained cytoplasm with from one to two nuclei and a regular outline. The elongated and ramified varieties showed no difference in comparison with those present in cultures of human connective tissue, with the exception that no mitotic figures were observed. Figure 3 shows a few of the peripheral cells in a culture of sarcomatous tissue which had been carried through twelve passages.

One culture was stained which was growing actively in its twelfth passage (twenty-one days). This culture is shown in figure 1, the area of cell proliferation being that which developed in the twelfth passage during forty-eight hours' incubation. One other culture was carried through for twenty-four passages, that is, fifty-two days. It was possible to divide this culture in its fifteenth passage, making two, and after forty-eight hours' incubation one of these cultures was fixed. Figure 2 shows almost the entire culture.

Sarcomatous tissue grew as well during a few days as normal connective tissue. Afterwards the rate of growth became less rapid and the tissue could not be kept alive for more than fifty-two days, while normal human tissue could be kept for sixty-eight days.

These differences may be due to technical factors, but they may also be the result of the nature of the tissue itself. In his attempts at keeping Rous sarcoma in a condition of permanent life *in vitro,* Carrel[6] observed that after a few generations the rate of growth became less rapid than the rate of growth of connective tissue. In

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[6] Carrel, A., *Jour. Exper. Med.,* 1912, xv, 516.

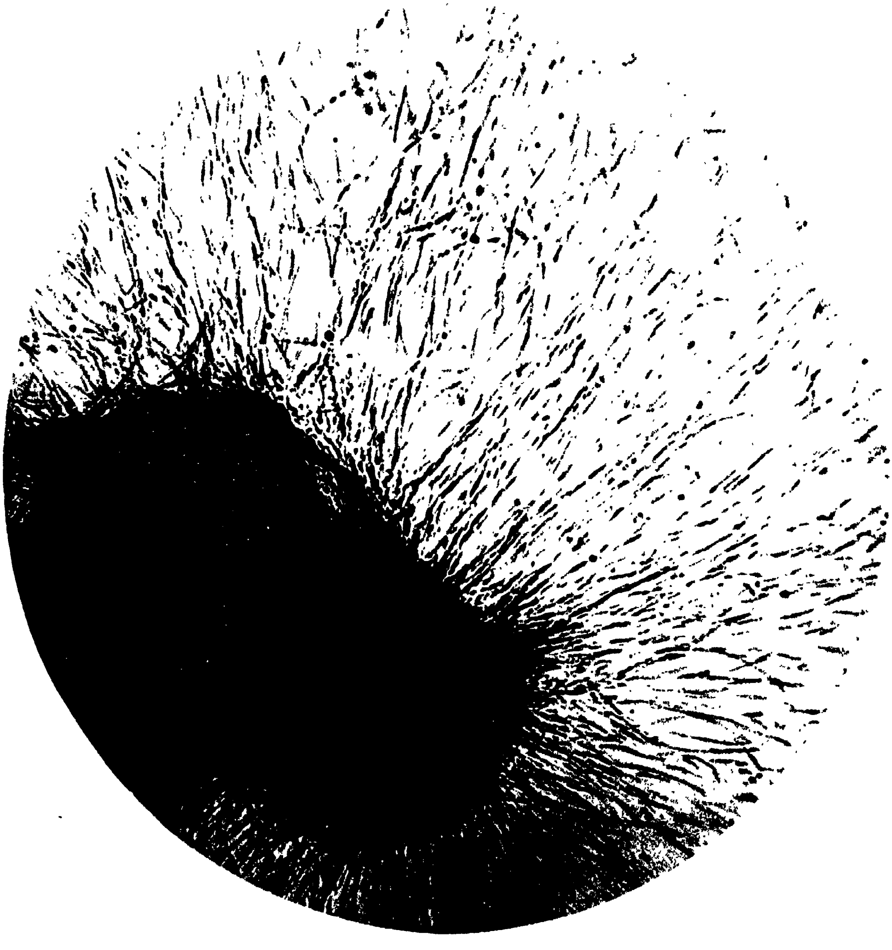

Fig. 1.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro*.)

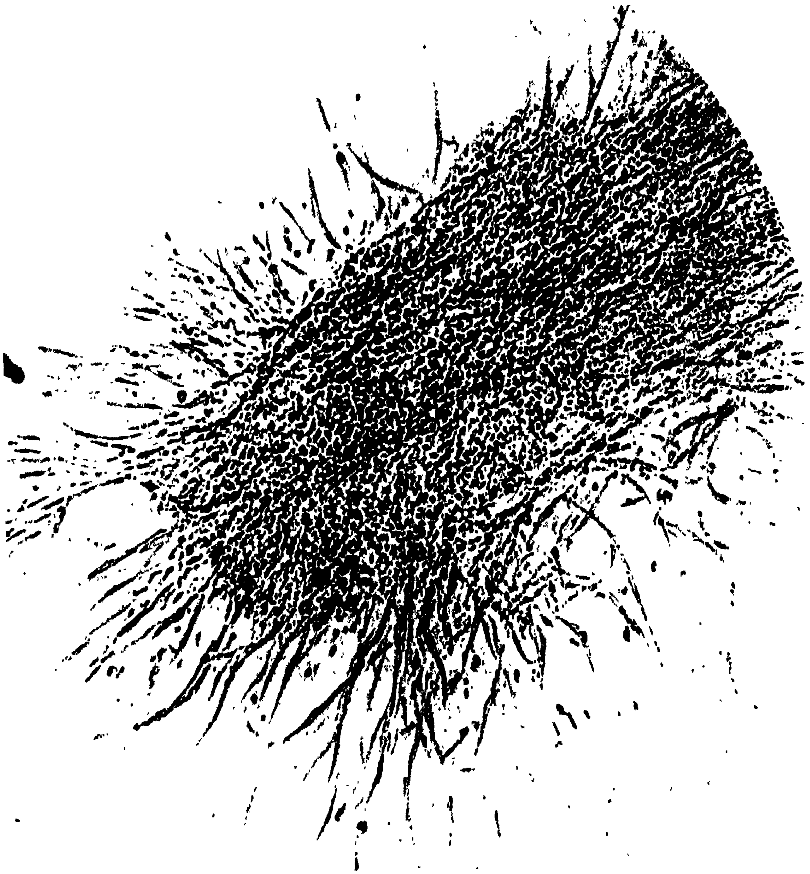

FIG. 2.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro*.)

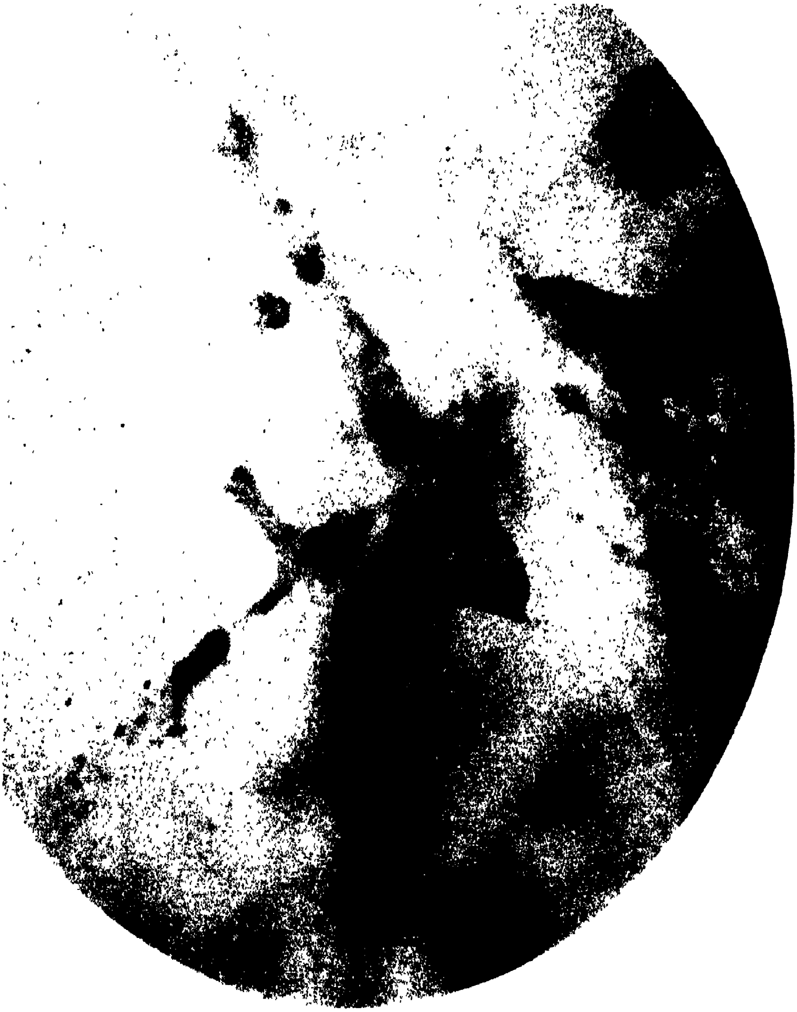

FIG. 3.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro*.)

other experiments with rat sarcoma and normal rat connective tissue, cultivated in guinea pig plasma, Carrel also observed the same differences. The writers observed the same phenomena when rat sarcoma and normal heart tissue of the rat were cultivated in chicken plasma.

The results obtained show that it is possible to cultivate *in vitro* fragments of human sarcomatous tissue for several generations, and that the method employed may prove of value in the study of the growth of human malignant tumor.

EXPLANATION OF PLATES.

PLATE 15.

FIG. 1. 12th passage of human sarcomatous tissue. The preparation shows the area of cell proliferation that developed during forty-eight hours' incubation. Giemsa stain.

PLATE 16.

FIG. 2. 15th passage of human sarcomatous tissue. The photograph shows the growth obtained forty-eight hours after passage into fresh medium. Fixed specimen.

PLATE 17.

FIG. 3. High power magnification of some of the peripheral cells present in the same preparation shown in figure 1.

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ON THE TECHNIQUE OF INTRATHORACIC OPERATIONS.

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Although the technique of intrathoracic surgery has been very much improved during the last few years, complications still often follow upon operations on the lungs, the œsophagus, and the heart. For this reason the technical details by means of which intrathoracic operations may be rendered more safe must be worked out. The purpose of this paper is to describe the methods by which the occurrence of pleurisy and of air emboli of the coronary arteries can be prevented.

It is probable that the development of pleurisy is always due to infection or irritation produced by the handling of the pleura with sponges or instruments, by the contact of the blood or by the germs of the air. Most experimenters and surgeons in their operations on the pleura employ the same technique which is used in abdominal surgery. They do not realize that the pleura is very much more liable to infection than the peritoneum and that the mechanical irritation, as well as certain factors of infection, such as the atmospheric germs, are possibly able to produce dangerous complications. Several years ago I read a paper before the members of this Society, in which that question was already investigated and in which the details of a technique for the prevention of these mild but very dangerous infections were described. At that time great care was taken in handling the intrathoracic organs, in sponging and walling off the operating field with gauze, and in protecting the pleura from the various factors which bring about irritation and infection. As soon as the thoracic cavity was opened the lungs were covered with fine silk compresses impregnated with vaseline. The silk tissue acted as a thin and almost impermeable membrane which protected the pleura without irritating it and permitted a very efficient walling off of the operating field to be effected. At the same time it prevented the evaporation and the desiccation of the surface. Of twelve animals operated upon at that time all twelve recovered. Nevertheless, after more complicated operations, such as the patching of the vena cava, the grafting of vessels onto the heart, some simultaneous operations on the heart and descending aorta, it was observed, when a large exposure of the thoracic cavity was rendered necessary, that serofibrinous or purulent pleurisy was eventually present. In 28 operations performed these complications occurred 7 times.

Although these experiments were made a long time ago, it appears that the technique for the prevention of pleurisy has not been further developed as yet. Both American and European surgeons are still describing more or less complicated methods for the drainage of the pleura. This evidently shows that pleurisy is still an ordinary complication accompanying intrathoracic operations. For this reason I have again attempted to ascertain whether in experimental operations this complication could be entirely avoided.

The use of Japanese silk towels impregnated with vaseline had already in a large measure diminished the danger of the occurrence of pleurisy; but it was nevertheless probable that the silk membrane did not sufficiently protect the remoter parts of the pleural cavity. Moreover, when hæmorrhage occurred the blood flowed along the greased surface of the pad and accumulated in the lower part of the thoracic cavity. For this reason the technique had to be modified, and this was effected in the following manner: The operating field was walled off by two kinds of towels. The first kind was made of Japanese silk which had been previously boiled in water, dried and sterilized in the autoclave, like ordinary pieces of dressing. The second kind was composed of absorbent cotton and of black Japanese silk. These towels were made in the following way: Two pieces of fine black Japanese silk were sewn together at the edges. Between these two pieces was placed a layer of absorbent cotton about one centimeter thick, and the whole towel was knotted throughout, thus forming a pad. These towels were sterilized in the autoclave. Both kinds of towel above described were used for walling off the operating field. When the incision of the superficial part of the thoracic wall was completed and the hæmostasis secured, the pleural cavity was opened by means of a small incision made in the middle of an intercostal space. A dry white Japanese silk towel was introduced into one end of the incision, while a second one was introduced at the other end. These towels afterwards served as a protection to the anterior and posterior parts of the pleural cavity. Next, the incision of the thoracic wall was completed and the thoracic cavity was opened wide, the lungs being meanwhile completely protected by the towels already introduced. Immediately after this the black silk and cotton padded towels were laid on the upper and lower edges of the wound and introduced into the cavity in such a manner that they respectively protected the upper and lower parts of the pleura. Next, a Gosset retractor was applied and the edges of the wound were retracted as much as was necessary for the purpose of the operation. The edges of the padded towels were arranged in such a way as to circumscribe the operating field and to leave this alone exposed to the air and to the sight of the operator. Additional padded towels could be used afterwards, if necessary, in order to secure a more complete walling off of the operating field. By means of this procedure the pleural cavity appeared to be almost completely protected against the infection produced by the atmospheric germs, as well as against all possible infection or irritation caused by the handling of the serous membrane by the hands of the operators, by the rough sponging with gauze, and by other operative traumatisms. Moreover, when hæmorrhage occurred the blood was prevented from flowing into other parts of the thoracic cavity.

The technique described above was recently used in eight operations of the patching of the pulmonary artery and the incision of the pulmonary orifices, in six operations of cauterization or incision and suturing of the sigmoid valves and pulmonary orifice, and in one operation of the cauterization of the sigmoid valve of the aorta. In two cases the animal died on the operating table, and in one case in which rubber gloves were not used for the handling of the heart and pericardium the animal died a few days later of pericarditis. The twelve other animals remained in good health. These results thus demonstrate that in extensive operations on the thoracic cavity, which are often accompanied by hæmorrhage from the heart, the pleura can be efficiently protected, and intrathoracic operations may consequently become as safe as operations on the abdominal cavity.

The general principles used in blood-vessel surgery are sufficient for most operations on the heart, and complications such as thrombosis or hæmorrhage can be completely prevented by employing a technique more or less similar to that used for the suturing of large blood-vessels. Nevertheless, there is one complication which often causes death in intracardiac operations and this is the occurrence of air emboli. When the heart is opened wide and closed again by suture it remains filled with air, and when the circulation is reëstablished the blood carries with it a number of air emboli. In operations performed on the right heart or on the pulmonary artery these air emboli did not appear to have any injurious effects; but after the left cavities of the heart or the aorta had been opened and the wall again closed by suture the reëstablishment of the circulation was always accompanied by the entrance of air emboli which were usually most dangerous. A more or less large quantity of air, according to the nature of the operation, entered the mouth of the coronary arteries and produced small air emboli in the terminal branches. They could easily be seen in the lumen of the vessels and appeared in the form of small air bubbles which filled the ramifications of the arteries. The circulation of the territory invaded by these air emboli was not reëstablished; the muscle remained bluish in color and the heart was unable to recover its normal pulsations. Usually fibrillary contractions occurred a short time after the reëstablishment of the circulation, and this was followed by the death of the animal. The above complications occurred every time the left ventricle or aorta was widely opened and the air was allowed to penetrate the cavities of the heart. It was evident that some procedure serving to prevent the occurrence of air emboli had to be developed before it would be possible to perform intracardiac operations on the left cavities of the heart. The method selected consisted in aspirating the air contained in the heart after the suture of the wall had been completed and before the forceps used to clamp the pedicle of the heart was removed. The aspiration was effected in two different ways. According to the first procedure a needle was introduced into the left ventricle from where the aspiration was effected. In the second instance a curved glass cannula was introduced through the line of suture of the aorta as far as the aortic orifice, and the air was aspirated. By using both these methods the heart could be emptied of air, and the production of air emboli after the reëstablishment of the circulation was prevented. As, however, this method of eliminating the air by means of aspiration was often imperfect, a further method had to be discovered whereby the occurrence of air emboli might not only be prevented but also remedied.

In cases where a great deal of air has entered the coronary arteries no treatment at all would be of any use. If most of the branches of the arteries are filled with air bubbles the fibrillary contractions appear almost immediately after the reëstablishment of the circulation, in which case it is probably too late to apply an efficient method of treatment. Nevertheless, it is not impossible that even in these cases the recovery of the heart can be effected if the air emboli can be removed. The technique to be described was only used in cases in which small amounts of air had been injected into the coronary branches. It is well known that the puncture of an arterial wall with a needle No. 16 is followed by hæmorrhage, but that this hæmorrhage always stops spontaneously after a short time. By introducing a needle No. 16 obliquely under the visceral pericardium and by perforating the wall of the coronary artery hæmorrhage was produced and the air was eliminated at the same time as the blood. This is but a slight sub-pericardial hæmorrhage, which is without danger and is easily arrested. But as soon as the air had been aspirated from the lumen of the artery a normal circulation through the coronary system was reëstablished and the heart immediately resumed its normal pulsations. The prevention of the occurrence of air emboli and their treatment adds greatly to the safety of the operations performed on the cavities of the heart.

It eliminates one of the chief causes of fibrillary contraction and subsequent death following upon intracardiac operations.

It is certain that these new methods of procedure do not suffice to eliminate all danger from intrathoracic operations; but at all events they add some important factors of safety to the technique of the operations of the heart and lungs.

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WEITERE UNTERSUCHUNGEN UBER DAS ELEKTRODENÄHNLICHE VERHALTEN WASSERUNMISCHBARER[1]) ORGANISCHER SUBSTANZEN.*

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MIT 4 FIGUREN IM TEXT.

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Die vorliegende Mitteilung befasst sich mit der experimentellen Untersuchung von Potentialdifferenzen, die an der Berührungsfläche wasserunmischbarer elektrolytisch leitender organischer Substanzen und wässeriger Salzlösungen auftreten, oder, mit andern Worten: mit den elektrizitätserregenden Kräften, welche durch Ketten aus solchen wasserunmischbaren Substanzen und Salzlösungen entstehen.

Wie vom Verfasser[2]) in einer frühern Mitteilung experimentell nachgewiesen wurde, sind diese E. K. von ähnlicher Grössenordnung wie diejenigen von Ketten aus Metallen und Salzlösungen, und damit ist eine Erklärung für das Zustandekommen der erheblichen E. K. gefunden, welche von lebenden Geweben hervorgerufen werden[3]).

Wichtiger noch als diese Ähnlichkeit in der Grössenordnung der E. K. ist die Analogie in dem Verhalten von Einzelpotentialdifferenzen gegen Konzentrationsänderungen: Metallelektrodenpotentialdifferenzen, Potentialdifferenzen an Geweben und solche an wasserunmischbaren reinen chemischen Substanzen zeigen eine ähnliche logarithmische Konzentrationsabhängigkeit. Die Beschreibung und physikalische Erklärung dieser Erscheinung bildete den Hauptgegenstand der genannten Abhandlung des Verfassers.

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* Eingegangen am 8. 1. 14.

[1]) Wasserunmischbar ist hier stets im Sinne von partiell unmischbar mit Wasser gebraucht.

[2]) Z. f. Elektroch. 19, 319 u. 467 (1913).

[3]) Betreffend die biologische Seite der Frage siehe J. Loeb und R. Beutner, Biochem. Zeitschr. 51, 288 (1913).

I. FRÜHERE UNTERSUCHUNGEN UND THEORIE DER PHASENGRENZPOTENTIALDIFFERENZEN.

Zahlreiche Untersuchungen liegen über die biologischen Potentialdifferenzen vor, und auch einige, die sich mit der künstlichen Nachahmung derselben mit Hilfe von organischen Substanzen1) befassen. Diese Forschungen berichten jedoch nichts über die Ähnlichkeit des elektromotorischen Verhaltens von wasserunmischbaren organischen Substanzen und Metallen, und zum Teil deshalb nicht, weil die einfachen thermodynamischen Schlussfolgerungen nicht berücksichtigt sind, auf Grund deren sich dies Verhalten ableiten lässt, die fundamentale Theorie der Phasengrenzpotentialdifferenzen, welche eben auch zwischen wasserunmischbaren organischen Substanzen und wässerigen Salzlösungen auftreten.

Diese Theorie lässt sich folgendermassen kurz darstellen. Jedes System:

Metall | elektrolytischer Leiter I | elektrolytischer Leiter II | Metall
1 2 3 (wie am Anfang)

worin die beiden elektrolytischen Leiter unmischbar sind und an der gemeinsamen Phasengrenze 2 im Gleichgewicht stehen, hat nach den Prinzipien der Thermodynamik die E. K. Null, weil arbeitleistende Veränderungen bei Stromfluss durch das System unmöglich sind. Die Summe der drei Potentialdifferenzen an den drei Unstetigkeitsstellen ist deshalb Null oder, anders ausgedrückt:

$$\text{P. D. } 2 = \text{P. D. } 1 + \text{P. D. } 3.$$

Ist c_1 die Konzentration der elektrolytischen Ionen im Leiter I, so gilt bekanntlich nach Nernst:

$$\text{P. D. } 1 = \frac{RT}{nF} \ln \frac{\text{konst.}}{c_1}.$$

Ist c_2 die Ionenkonzentration im Leiter II, so gilt:

$$\text{P. D. } 3 = \frac{RT}{nF} \ln \frac{c_2}{\text{konst.}},$$

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1) Hiermit sind besonders Arbeiten physiologischer Autoren gemeint. Besonders bemerkenswert ist M. Cremers Untersuchung, Zeitschr. f. Biol. 47, 562 (1906).

hieraus folgt:

$$\text{P. D. } 2 = \frac{RT}{nF} \ln \frac{c_1}{c_2} \cdot \text{konst.}$$

(elektromotorische Phasengrenzregel.)

Den wichtigen Untersuchungen Habers ist die wertvollste Aufklärung über die Natur der Phasengrenzpotentialdifferenzen zu danken. Haber[1]) zeigte, dass wasserunlösliche feste Salze an ihrer Grenzfläche gegen wässerige Lösungen eine P. D. entwickeln, welche die gleiche Konzentrationsabhängigkeit wie P. D. an Metallen zeigt. Untersucht wurde z. B. die P. D.: $\underset{\text{fest}}{AgCl} \mid AgNO_3$-Lösung. Dieselbe verhält sich bezüglich der Konzentrationsabhängigkeit wie die P. D. $Ag \mid AgNO_3$-Lösung[2]).

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[1]) Ann. d. Physik [4] 26, 947 (1908).

[2]) Nernst hatte allerdings schon vor längerer Zeit, von theoretischen Überlegungen ausgehend, für Phasengrenz-P. D. die oben abgeleitete Formel aufgestellt [Zeitschr. f. physik. Chemie 9, 385 (1892)]. Diese Formel umfasst auch die von Haber gemessenen P. D., man kann indes nicht sagen, dass die durch die Haberschen Untersuchungen realisierte „Metallähnlichkeit" der Phasengrenz-P.D. durch die Nernstsche Phasengrenzformel hätte leicht vorausgesagt werden können. Eine metallähnliche Veränderlichkeit der Phasengrenz-P. D. ist nach Nernsts Formel nur dann zu erwarten, wenn die Konzentrationen des gemeinsamen Ions in den beiden Phasen nicht einander proportional sind. In der Regel ist dies jedoch der Fall, wenigstens ist es auf Grund des Verteilungssatzes zu erwarten, wenn ein Elektrolyt im Gleichgewicht zwischen zwei Phasen verteilt ist. Die Konzentration des Undissociierten in den beiden Phasen ist einander proportional und, da in jeder Phase die Konzentration eines Ions ins Quadrat dem Undissociierten proportional ist, folgt, dass auch die Ionenkonzentration in beiden Phasen proportional ist. Bei dem von Haber untersuchten Beispiel: $AgCl \mid AgNO_3$-Lösung ist dies natürlich ganz anders; die (unbekannte) Ionenkonzentration des festen $AgCl$ wird in keiner Weise durch die $Ag^{\cdot}$-Konzentration der berührenden wässerigen Lösung beeinflusst; nach der Nernstschen Phasengrenzformel ist also:

$$E = \frac{RT}{nF} \ln c_{Ag^{\cdot}} . \text{konst.},$$

wie an einer Ag-Elektrode. Habers Untersuchungen haben daher zuerst die charakteristische Ähnlichkeit von Phasengrenz-P.D. und Elektroden-P.D. dargetan und damit der Fragestellung bei weitern Forschungen die richtigen Gesichtspunkte gegeben.

2. FRÜHERE UNTERSUCHUNGEN DES VERFASSERS DER KONZENTRATIONSVERÄNDERLICHKEIT DER POTENTIALDIFFERENZEN AN ORGANISCHEN SUBSTANZEN.

Der Fortschritt, den die Untersuchungen des Verfassers herbeigeführt haben, betrifft die physikalische Erklärung der Reversibilität für verschiedene Ionen gleichen Vorzeichens, welche nach Untersuchungen von J. Loeb und dem Verfasser für das elektromotorische Verhalten von Geweben kennzeichnend ist. Organische Gewebe zeigen nämlich bei Berührung mit wässerigen Salzlösungen eine elektrodenähnliche Veränderlichkeit der P. D., gleichgültig, welches Salz in der wässerigen Salzlösung enthalten ist. Ein derartiges Verhalten ist nach den beschriebenen Haberschen Untersuchungen und der elektromotorischen Phasengrenzregel nicht leicht zu erklären. Es gelang nun aber dem Verfasser, zu zeigen, dass eine ganze Anzahl reiner organischer Substanzen ebenfalls eine solche Reversibilität für verschiedene Ionen zeigen, nicht nur die Kationenreversibilität, die für organische Gewebe kennzeichnend ist, sondern auch unter Umständen eine Änderung in der entgegengesetzten Richtung (Anionenreversibilität). Durch systematische Untersuchungen dieser neuen galvanischen Erscheinungen an organischen Substanzen wurden wichtige Aufschlüsse über ihre physikalische Natur gewonnen und gezeigt, in welcher Weise sie auf Grund der elektromotorischen Phasengrenzregel zu erklären sind.

Ein kurzes Resümee meiner frühern Arbeit wird am besten durch Beschreibung des wichtigsten Experiments gegeben. Dieselbe experimentelle Methode wurde auch bei allen hier neu beschriebenen Versuchen verfolgt.

Eine ∾-förmig gebogene Röhre (siehe Figur 1) wird mit der schmaleren Öffnung in eine Normalelektrode eingeführt und ganz mit der Elektrodenlösung (meist 1/1 KCl) gefüllt. Durch die weite Öffnung wird dann die Lösung durch Salicylaldehyd (gesättigt an Salicylsäure) gefüllt. Zur Ausführung der Messungen werden verschieden konzentrierte KCl-Lösungen hergestellt, z. B. in den molaren Konzentrationen 1/2, 1/10, 1/50, 1/250, 1/1250, und die mit Salicylaldehyd gefüllte Röhre, sowie die kapillare Spitze einer zweiten Normalelektrode nacheinander in die verschieden konzentrierten Lösungen eingetaucht, und so für jede Konzentration eine Messung der E. K. ausgeführt[1]). Momentan stellt sich für jede Konzentration eine charakteristische E. K. ein, welche nahezu konstant bleibt. Beim Wiedereintauchen in die zuerst benutzte Lösung kehrt die gleiche E. K. momentan wieder zurück. Die Differenzen dieser E. K. sind also jeweils die Kräfte reversibler Konzentrationsketten. Folgende Tabelle aus einer frühern Arbeit lässt den erreichten Grad von Genauigkeit und die zeitliche Reversibilität erkennen.

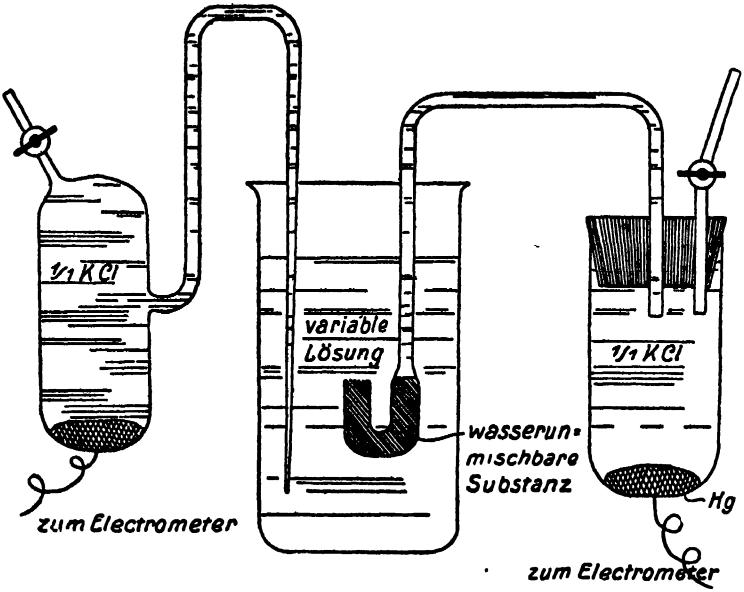

Fig 1.

Die physikalisch-chemische Natur der P. D. erkennt man, wenn man an Stelle des Salicylaldehyds andere wasserunmischbare elektrolytisch leitende Substanzen als Mittelleiter verwendet. Es zeigt sich dann, dass die sauren Eigenschaften der Salicylsäure, welche im Salicylaldehyd enthalten ist, etwas mit der Veränderlichkeit der P. D. zu tun haben, denn Phenol,

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[1]) Als Messinstrument wurde das Dolezaleksche Binantenelektrometer benutzt (Ablesung mit Spiegel und Skala).

P. D.: Salicylaldehyd, gesättigt an Salicylsäure. *KCl* in Wasser.

Zeit in Minuten.	Konzentration der *KCl*-Lösung in g-Molen.	Millivolt.	Positiver mit sinkender Konzentration um Millivolt.
0	1/10	+ 12	
1	1/10	+ 13	24
3	1/50	+ 37	
4	1/50	+ 37	25
7	1/250	+ 62	
9	1/250	+ 62	34
11	1/1250	+ 96	
12	1/1250	+ 96	41
17	1/6250	+ 137	
19	1/6250	+ 137	41
20	1/1250	+ 96	
23	1/1250	+ 95	34
26	1/250	+ 61	
73	1/250	+ 60	
78	1/50	+ 32	28
81	1/50	+ 31	
84	1/10	+ 7	24
86	1/10	+ 7	
88	1/2	− 14	21
90	1/2	− 14	
95	5/2	− 32	18
97	5/2	− 33	
103	1/2	− 15	18
104	1/2	− 15	
107	1/10	+ 7	22
108	1/10	+ 7	

Kresole, höhere Alkohole u. a. zeigen keine oder nur eine geringe Veränderlichkeit der P. D., Basen wie Anilin, Naphtylamin und deren Substituenten zeigen eine Veränderlichkeit mit der Konzentration in der entgegengesetzten Richtung.

Messungen mit Mischungen von wasserunlöslichen Basen und Säuren als Mittelleiter und wässerigen Lösungen von Salzen wie Natriumbenzoat oder Salicylat oder Anilinchlorhydrat, Dimethylanilinchlorhydrat o. a. haben Aufklärung darüber gebracht, in welcher Weise die Kationenreversibilität der wasserunmischbaren Säuren und die Anionenreversibilität der wasserunmischbaren Basen zustande kommt[1]). Das Resultat ist beispielsweise für die Salicylaldehyd-P. D.: zwischen der Salicylsäure (im Aldehyd) und *KCl* findet spurenweise eine Umsetzung statt, wobei *HCl* und *K*-Salicylat gebildet wird; das letztere Salz reichert sich im Aldehyd an bis zu einer Konzentration, die gleich oder grösser wie die des *KCl* ist; hierdurch wird die Nichtproportionalität der *K'*-Konzentration in Aldehyd und Wasser ermöglicht, was nach der zitierten elektromotorischen Phasengrenzformel die Vorbedingung für die Konzentrationsveränderlichkeit der P. D. ist.

3. POTENTIALDIFFERENZEN AN WASSERUNMISCHBAREN SUBSTANZEN, HERVORGERUFEN DURCH ZWEI GLEICHKONZENTRIERTE WÄSSERIGE LÖSUNGEN VERSCHIEDENER ELEKTROLYTE.

Der eigentliche Gegenstand der vorliegenden Arbeit betrifft chemische Einflüsse bei solchen P. D. Anstatt einer Reihe verschieden konzentrierter Lösungen desselben Elektrolyts werden jetzt molekular gleich konzentrierte Lösungen verschiedener Elektrolyte untersucht. Obzwar nämlich verschiedene Elektrolyte eine gleiche Konzentrationsveränderung der P. D. hervorrufen können, ist der Wert der P. D. bei gleichen Konzentrationen verschiedener Elektrolyte in der Regel verschieden.

Wir verwenden wieder den Apparat wie auf Seite 422 beschrieben, das untere weite U-Rohr mit Salicylaldehyd gefüllt, und haben in Bechergläsern von je 100 ccm folgende Lösungen:

$$m/10\ KCl,\ m/10\ NH_4Cl,\ m/10\ BaCl_2,\ m/10\ MgCl_2.$$

Taucht man die Röhre mit Salicylaldehyd und eine zweite ableitende Elektrode in diese Lösungen, so beobachtet man nacheinander folgende E. K.[2]).

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[1]) Näheres hierüber siehe loc. cit. [Z. f. Elektroch. 19, 322 (1913)]. Aus Raummangel können diese Experimente hier nicht wieder beschrieben werden, obgleich sie für die Theorie die wichtigsten sind.

[2]) Früher mitgeteilte Messungen dieser P. D. (loc. cit.) weichen von diesen Daten ab, da Salicylaldehyd bei diesen Versuchen nicht an Salicylsäure gesättigt ist.

Zeit in Minuten.	Lösung.	E. K.	Zeit in Minuten.	Lösung.	E. K.
0′	$m/10\ KCl$	+ 0.039 Volt	14′	$m/10\ MgCl_2$	+ 0.114 Volt
2′	"	+ 0.039 "	16′	"	+ 0.110 "
4′	$m/10\ NH_4Cl$	+ 0.052 "	18′	$m/10\ CaCl_2$	+ 0.103 "
6′	"	+ 0.051 "	20′	"	+ 0.103 "
8′	$m/10\ BaCl_2$	+ 0.090 "	22′	$m/10\ KCl$	+ 0.039 "
10′	"	+ 0.093 "			

Diese Zahlen zeigen, dass wie beim Konzentrationseffekt die P. D. zeitlich völlig reversibel ist. Die Differenz zwischen beispielsweise den letzten beiden Messungen kann als reversible E. K. der Kette

$$- 1/10\ KCl \mid \text{Salicylaldehyd} \mid 1/10\ CaCl_2 + \qquad (I)$$

angesehen werden, 0.064 Volt.

Wie kann die E. K. einer solchen Kette im voraus berechnet werden? Offenbar ist dieses Problem komplizierter wie die Berechnung einer Konzentrationskette

$$1/10\ KCl \mid \text{Salicylaldehyd} \mid 1/100\ KCl \qquad (II)$$

denn der chemische Charakter der beiden Salze ruft die ersten E. K. hervor.

Zwei Methoden zur Berechnung einer solchen „chemischen" Kette scheinen anwendbar.

4. ERKLÄRUNG DER CHEMISCHEN E. K. DURCH BESTIMMUNG EINER ENTSPRECHENDEN REAKTION. REGEL VON DER UNABHÄNGIGKEIT.

In meiner frühern Untersuchung wurden diese „chemischen" E. K. ebenfalls erwähnt, und es wurde eine Erklärung vorgeschlagen, die im Prinzip auf folgendes hinaus läuft. Eine Kette wie z. B. Kette (I) enthält im Salicylaldehyd an der Grenze gegen die KCl-Lösung K-Salicylat, (wie sich aus der Änderung der P. D. auf Grund der elektromotorischen Phasengrenzregel hatte folgern lassen), an der Grenze gegen die $CaCl_2$ Ca-Salicylat, also:

$$-KCl \text{ in Wasser} \mid \underbrace{K\text{-Salicylat} \mid Ca\text{-Salicylat}}_{\text{in Salicylaldehyd}} \mid CaCl_2 \text{ in Wasser} + \qquad (I)$$

Die Kette ist also früher untersuchten Ketten[1]) in gewisser Hinsicht analog, wie z. B. der Kette:

$$+\,Ag \;\Big|\; \underset{\text{fest}}{Ag_2SO_4} \;\Big|\; \underset{\text{fest}}{Na_2SO_4} \;\Big|\; \underset{\text{fest}}{NaCl} \;\Big|\; \underset{\text{fest}}{AgCl} \;\Big|\; Ag\,- \qquad \text{(II)}$$

Die E. K. dieser letztern Kette, 0.532 Volt, rührt daher, dass bei Stromfluss die Reaktion vor sich geht:

$$Ag_2SO_4 + 2\ NaCl = 2\ AgCl + Na_2SO_4{}^{2})$$

Analog ist für Kette (I):

$$CaCl_2 + K\text{-Salicylat} = Ca\text{-Salicylat} + KCl$$

Dass eine praktische Anwendung dieser Berechnungsmethode unmöglich ist, ist leicht zu sehen und wurde vom Verfasser schon in der frühern Abhandlung hervorgehoben: erstens nämlich lässt sich nicht wie beim Daniellelement die stromliefernde Reaktion durch Beobachtung der Veränderungen bei Stromfluss direkt realisieren; ferner ist auch die Wärmetönung der Reaktion nicht bekannt, und schliesslich sind die Ketten (I) und (II) nicht in jeder Beziehung elektromotorisch analog.

Besondere Schwierigkeiten bietet die Anwendung der Theorie bei Ketten mit solchen Mittelleitern, die keine Konzentrationsabhängigkeit der P. D. zeigen. Ob eine Umsetzung an den Phasengrenzen eintritt, kann in solchen Fällen nicht mehr direkt bewiesen werden, man kann aber hieraus nicht etwa schliessen, dass diese Substanzen auch keine „chemischen" E. K. hervorrufen können. Die Beobachtungen ergeben denn auch, dass die Grösse der Konzentrationsveränderlichkeit der P. D. zu den „chemischen" E. K. in keiner Beziehung steht. Beispielsweise zeigt o. Kresol nur eine geringe Konzentrationsveränderlichkeit:

$$-\,1/10\ KCl \;|\; \text{o. Kresol} \;|\; 1/1000\ KCl + 0.007\ \text{Volt}$$

während Salicylaldehyd als Mittelleiter ergibt:

$$-\,1/10\ KCl \;|\; \text{Salicylaldehyd} \;|\; 1/1000\ KCl + 0.08\ \text{Volt.}$$

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[1]) Zeitschr. f. Elektrochem. 13, 433, 1909.

[2]) Hierfür waren mehrere Beweise möglich, siehe l. c.

Trotzdem ist die E. K. der Ketten:

$+\ 1/10\ KCl$ | o. Kresol | $1/10$ Dimethylanilin HCl —

und

$+\ 1/10\ KCl$ | Salicylaldehyd | $1/10$ Dimethylanin HCl —

etwa gleich gross: 0.08 Volt.

Die andere Erklärungsmethode, welche hier gegeben werden soll, erklärt diese Beobachtungen leicht; trotzdem ist es nicht möglich, die obige Theorie gänzlich bei Seite zu lassen, weil es ein elektromotorisches Phänomen gibt, welches anders nicht thermodynamisch abgeleitet werden kann. Es ist dies die Regel von der Unabhängigkeit der Ionenwirkung.

Die beobachteten Erscheinungen sind folgende: In der Apparatur, die oben beschrieben ist, wird die U-Röhre z. B. mit Guajakol als wasserunmischbaren Mittelleiter gefüllt; gleichkonzentrierte Lösungen mehrerer Serien von Salzen werden so hergestellt, dass alle Salze jeder einzelnen Serie immer ein gemeinsames Ion haben. Die U-Röhre wird nacheinander in diese Lösungen eingetaucht, und die E. K. in jedem Falle beobachtet (mehrere Messungen bis zur Konstanz).

		Differenz.			Differenz.
$m/10\,KCl$	$+\,0.010$ Volt		$m/10\,NaCl$	$+\,0.020$ Volt	
		$>$ 0.025 Volt			$>$ 0.025 Volt
$m/10\,KNO_3$	$+\,0.035$ "		$m/10\,NaNO_3$	$+\,0.045$ "	
		$>$ 0.048 "			$>$ 0.046 "
$m/10\,K_2SO_4$	$-\,0.013$ "		$m/10\,Na_2SO_4$	$-\,0.001$ "	
		$>$ 0.081 "			$>$ 0.078 "
$m/10\,KSCN$	$+\,0.068$ "		$m/10\,NaSCN$	$+\,0.077$ "	

		Differenz.			Differenz.
$m/10\,KCl$	$+\,0.010$ Volt		$m/10\,KNO_3$	$+\,0.035$ Volt	
		$>$ 0.011 Volt			$>$ 0.011 Volt
$m/10\,NaCl$	$+\,0.021$ "		$m/10\,NaNO_3$	$+\,0.046$ "	
$m/10\,CaCl_2$	$+\,0.074$ "	$>$ 0.053 "	$m/10\,Ca(NO_3)_2$	$+\,0.099$ "	$>$ 0.053 "
$m/10\,HCl$	$+\,0.023$ "	$>$ 0.051 "	$m/10\,HNO_3$	$+\,0.048$ "	$>$ 0.051 "
$m/10$ Dimethylanilin HCl	$-\,0.071$ "	$>$ 0.094 "	$m/10$ Dimethylanilin HNO_3	$-\,0.046$ "	$>$ 0.094 "

Diese Messungen zeigen, dass die Änderung der P. D. bei Übergang zu einer andern Salzlösung, durch die Natur des beiden Salzen gemeinsamen Ions nicht beeinflusst wird, oder, mit andern Worten: Die Potentialdifferenz setzt sich additiv aus den elektromotorischen Wirkungen der Ionen zusammen.

Um zu zeigen, dass sich diese Erscheinung auf Grund der oben entwickelten Theorie voraussehen lässt, wenden wir die Theorie z.

B. auf die Ketten an

$$-m/10\ KCl \mid \text{Guajakol} \mid m/10\ CaCl_2 +$$

und

$$-m/10\ KNO_3 \mid \text{Guajakol} \mid m/10\ CaNO_3 +$$

Die E. K. ist in beiden Fällen 0.053 Volt. Da Guajakol schwach saure Eigenschaften besitzt, enthält es in Berührung mit Kaliumsalzlösungen eine Spur von Kaliumguajakol in Berührung mit *Ca*-Salzlösungen Spuren von Calciumguajakol (analog wie bei Salicylaldehyd). Die den beiden Ketten entsprechenden Reaktionen sind also nach der Theorie:

$$CaCl_2 \text{ in Wasser} + \underset{\text{in Guajakol}}{K\text{-Guajakol}} = KCl \text{ in Wasser} + \underset{\text{in Guajakol}}{Ca\text{-Guajakol}}$$

$$Ca(NO_3)_2 \text{ in Wasser} + \underset{\text{in Guajakol}}{K\text{-Guajakol}} = KNO_3 \text{ in Wasser} + \underset{\text{in Guajakol}}{Ca\text{-Guajakol}}$$

Vorausgesetzt, dass die *K*-, resp. *Ca*-Guajakolkonzentrationen in beiden Fällen gleich sind, ist die freie Energie dieser Reaktionen gleich, denn die Differenzreaktion:

$$KCl + NaNO_3 = NaCl + KNO_3$$

in wässeriger Lösung hat die freie Energie Null, da alle Salze praktisch völlig elektrolytisch dissociiert sind.

Analog ist der Beweis für die beiden Salzreihen mit gemeinsamem Anion zu führen, wobei man allerdings zu der Annahme greifen muss, dass eine Substanz wie Guajakol sich auch mit Säuren verbindet, also amphotere Eigenschaften hat.

Mit andern wasserunmischbaren Substanzen an Stelle von Guajakol können dieselben Beobachtungen gemacht werden. Es sind jedoch auch Ausnahmen von der Regel vorhanden, was nach der Ableitung zu erwarten ist, da Bedingungen vorausgesetzt sind, die nicht immer notwendig erfüllt sind. Anormal dissociierte Salze wie z. B. $HgCl_2$ geben bisweilen starke Abweichungen. Beispielsweise wurde gemessen mit Acetophenon als Mittelleiter:

			Differenz.
$m/10\ NaCl$		$+0.039$ Volt	> 0.030 Volt
$m/10\ NaNO_3$		$+0.069$ "	
$m/10\ CaCl_2$		$+0.085$ "	> 0.030 "
$m/10\ Ca(NO_3)_2$		$+0.115$ "	
$m/10\ Hg(NO_3)_2$		$+0.060$ "	
$m/10\ HgCl_2$	ber.	$+0.030$ "	
	beobachtet	$+0.125$ "	

5. ERKLÄRUNG DER CHEMISCHEN E. K. DURCH VERSCHIEDENHEIT DER TEILUNGSKOEFFIZIENTEN DER BEIDEN SALZE.

Unser Ziel muss es sein, die Grösse einer chemischen E. K. aus Beobachtungen anderer Natur herzuleiten. Da die beschriebene Theorie in dieser Hinsicht nicht zu praktischen Ergebnissen führen kann, empfiehlt sich eine andere Methode; die E. K. wird aus der Verteilung der beiden Salze im Gleichgewicht zwischen den beiden Phasen berechnet.

Folgendermassen können solche Berechnungen aus Verteilungsgrössen hergeleitet werden. In erster Linie ist es für die Richtung der E. K. von Bedeutung, ob die beiden Salze in der wässerigen Lösung ein gemeinsames Anion haben, oder ob sie ein gemeinsames Kation haben.

Nehmen wir etwa das erstere an, und berechnen wir z. B. die E. K. einer Kette wie

$$+\ m/10\ NaCl \mid \text{Guajakol} \mid m/10\ KCl\ -$$

Auf Grund der elektromotorischen Phasengrenzregel stellen wir die E. K. des Systems als Cl'-Konzentrationskette dar, gleich $\frac{RT}{nF} \lg \frac{c_1 c_2}{c_3 c_4} \cdot$ konst.

(Hierin ist c_1 die Cl'-Konzentration in 1/10 $NaCl$
c_3 „ „ „ 1/10 KCl
c_2 „ „ , welche sich im Guajakol beim Schütteln mit 1/10 $NaCl$-Lösung im Gleichgewicht herstellt
c_4 die Cl'-Konzentration in Guajakol im Gleichgewicht mit 1/10 KCl-Lösung. Die selbstverständliche Voraussetzung ist gemacht, dass an den Phasengrenzen Gleichgewicht herrscht.)[1])

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[1]) Es ist ferner die Annahme gemacht, dass nur geringe Diffusionspotentiale innerhalb des Guajakols auftreten; die Berechtigung dieser Annahme gründet sich auf Versuche, die an anderer Stelle besprochen werden sollen.

Da die Cl'-Konzentration in 1/10 $NaCl$ und 1/10 KCl ($c_1 = c_3$) gleich ist, folgt dass die E. K. gleich ist:

$$\frac{RT}{nF} \ln \frac{c_2}{c_4} \cdot \text{konst.}$$

Die Cl'-Konzentrationen, die sich im Guajakol im Gleichgewicht mit der wässerigen Lösung einstellen, hängen nun erstens von dem Dissociationsgrad des betr. Salzes in dem nichtwässerigen Lösungsmittel und dem Verteilungskoeffizient des Salzes zwischen Guajakol und Wasser ab. Allgemein kann man folgern, dass die Seite der Kette negativ ist, auf welcher Salz mit dem grössern Verteilungskoeffizienten Guajakol: Wasser, resp. mit der grössern Dissociation in Guajakol sich befindet.

Analoge Schlussfolgerungen sind für eine Kette möglich, bei welcher die beiden Salzlösungen ein gemeinsames Kation besitzen, jedoch ist naturgemäss die Richtung der E. K. umgekehrt, diejenige Seite der Kette, auf welcher sich das Salz mit dem grössern Teilungskoeffizient: $\frac{\text{Konzentr. in Guajakol}}{\text{Konzentr. in Wasser}}$ befindet, ist positiv. Schliesslich lässt sich mittels solcher Betrachtungen die E. K. selbst dann berechnen, wenn die beiden Salze überhaupt kein Ion gemeinsam haben. Beispielsweise zur Berechnung der E. K. der Kette

1/10 KCl | Guajakol | 1/10 Na_2SO_4

könnte man eine Schicht von 1/10 $NaCl$ in wässeriger Lösung in das Guajakol eingeschoben denken, wodurch die E. K. nicht beeinflusst werden kann. Die E. K. der Teilketten

1/10 KCl | Guajakol | 1/10 $NaCl$ und 1/10 $NaCl$ | Guajakol | 1/10 Na_2SO_4

wäre wie oben zu bestimmen.

Eine quantitative Anwendung dieser Theorie war allerdings bisher noch nicht möglich, da die Verteilung von Elektrolyten zwischen Wasser und den andern Lösungsmitteln nicht bekannt ist. Die Bestimmung dieser Grösse ist nicht ganz einfach, da im Verteilungsgleichgewicht stets die Salzkonzentration im organischen Lösungsmittel äusserst gering ist. (Hierauf beruht ja auch die rasche zeitliche Reversibilität der Potentialdifferenzen.) Ferner ist über die elektrolytische Dissociation in dem nichtwässerigen Lösungsmittel nichts bekannt. Bestimmungen dieser Grössen vorzunehmen, um mittels derselben die Theorie zu bestätigen, war vor der Hand dem Verfasser noch nicht möglich; trotzdem lässt sich aus den Beobachtungen über die Potentialdifferenz allein eine qualitative Bestätigung der Theorie entnehmen. Misst man nämlich die Potentialdifferenz an der Phasengrenze mehrerer wasserunmischbarer Substanzen und mehrerer Salzlösungen, so findet man, dass in allen Fällen eine salzsaure Lösung einer organischen Base (wie Anilin usw.) eine negative P. D. hervorruft, eine Lösung des Alkalisalzes einer organischen Säure eine positive P. D., wenn der Wert für $m/10$ $NaCl$ als willkürliche Null-P. D. gewählt wird. Beispielsweise ist für sämtliche Ketten:

$+\, m/10\, NaCl$ | Organ. Substanz | $m/10$ Anilinhydrochlorid —(III)

die Richtung E. K. die gleiche (wie durch Vorzeichen bezeichnet) und ebenso für sämtliche Ketten:

$-\, m/10\, NaCl$ | Organ. Substanz | $m/10$ Na-Salicylat $+$ (IV)

Die chemische Natur des Mittelleiters hat nur auf Grössenordnung der E. K. nicht auf die Richtung einen Einfluss. Dieses Verhalten ist somit ganz verschieden von dem der Konzentrationsketten mit organischen Mittelleitern, bei denen auch die Richtung der E. K. je nach der Natur der Mittelleiter variiert. (Die experimentellen Daten über P. D. Messungen mit verschiedenen organischen Substanzen und Salzlösungen sind am Schluss der Arbeit tabellarisch zusammengestellt.)

Aus der Richtung der E. K. bei einer Kette wie (III) folgt im Sinne der Theorie, dass für Anilinhydrochlorid der Verteilungskoeffizient (Konz. in organ. Subst.: Konz. in Wasser) grösser ist als für $NaCl$, da in diesem Falle die Salze das Anion gemeinsam haben. Bei Kette (IV) (gemeinsames Kation) folgt bei Anwendung der Theorie, dass Natriumsalicylat den grössern Verteilungskoeffizienten hat. Also für alle Salze, welche eine Basis oder eine Säure organischer Provenienz enthalten, wäre der genannte Verteilungskoeffizient grösser als für rein „anorganische“ Salze. Diese Folgerung darf wohl als plausibel bezeichnet werden.

Folgende Versuche mit verschieden substituierten Aminen zeigen, dass die P. D. um so negativer ist, je höher substituiert das Amin ist.

Potentialdifferenz an Benzaldehyd in Berührung mit folgenden Lösungen:

$m/10$ Monoäthylamin	HCl	—0.014 Volt
$m/10$ Triäthylamin .	HCl	—0.071 “
$m/10$ Tetraäthylamin	HCl	—0.089 “

Jede Ablesung $2'$ konstant. P. D. mit $m/10$ $NaCl$ willkürlich gleich 0.0 Volt gesetzt. Derselbe Versuch mit Anisaldehyd als Mittelleiter ergab:

$m/10$ Monoäthylamin	HCl	—0.023 Volt
$m/10$ Triäthylamin	HCl	—0.058 “
$m/10$ Tetraäthylamin	HCl	—0.066 “

Aus den P. D. Messungen mit verschieden substituierten Anilinhydrochloriden, welche man in tabellarischer Zusammenstellung am Schluss der Arbeit findet, kann man ebenfalls entnehmen, dass die P. D. um so negativer ist, je höher die Substitution ist.

Hieraus würde nach der Theorie folgen, dass der Verteilungskoeffizient (Aldehyd: Wasser) um so grösser ist, je mehr C-Atomgruppen das Aminchlorhydrat enthält. Auch hierüber liegen Messungen noch nicht vor, da jedoch bekannt ist, dass z. B. der Verteilungskoeffizient verschiedener Alkohole (Öl: Wasser) mit dem C-Gehalt steigt, dürfte etwas ähnliches für Aminchlorhydrate ebenfalls gelten.

Aus den tabellarisch zusammengestellten Messungsergebnissen ergibt sich ferner, dass solche organische Flüssigkeiten, welche Säuren gelöst enthalten und dementsprechend „Kationenreversibilität“ zeigen, besonders grosse E. K. mit Salzen verschiedener Kationen hervorrufen können, mit Salzen verschiedener Anionen nur kleine E. K. Wasserunlösliche Basen dagegen zeigen das entgegengesetzte Verhalten.

Salicylaldehyd z. B. (welcher stets Salicylsäure enthält) gibt in der Reihe KCl, $NaCl$, $CaCl_2$ grössere Differenzen der P. D. als in der Reihe $NaCl$, $NaBr$, NaJ, $NaSCN$. Umgekehrt bei o. Toluidin.

Diese Beobachtungen bestätigen die früher entwickelten Theorien des Verfassers (siehe Seite 389). Salicylaldehyd verhält sich in Berührung mit *Na*-Salzlösungen ähnlich wie eine *Na*-Elektrode. Daher hat die Kette

$$-\, m/10\ Na_2SO_4 \mid \text{Salicylaldehyd} \mid m/10\ NaSCN +$$

nur eine kleine E. K. : 0.018 Volt. o. Toluidin in Berührung mit Chloriden, ähnlich einer *Cl'*-Elektrode. Daher:

$$+\, m/10\ CaCl_2 \mid o\text{-Toluidin} \mid m/10\ NaCl - 0.010 \text{ Volt.}$$

Dagegen:

$$-\, m/10\ Na_2SO_4 \mid o\text{-Toluidin} \mid m/10\ NaSCN + 0.260 \text{ Volt}$$

und:

$$+\, m/10\ CaCl_2 \mid \text{Salicylaldehyd} \mid m/10\ NaCl - 0.064 \text{ Volt.}$$

Diese Phänomene sollen später an anderer Stelle eingehend erörtert werden.

Die Versuche in Kolumne 7, 8 und 9 zeigen, dass Mischungen verschiedener wasserunmischbarer Substanzen im allgemeinen elektromotorische Erscheinungen zeigen wie reine Substanzen.

Die Beobachtung, dass *KCl* stets eine negativere P. D. als *NaCl* gibt, hat eine besondere biologische Bedeutung[1]).

6. VERSCHIEDENHEIT DER ZEITLICHEN REVERSIBILITÄT DER POTENTIALDIFFERENZEN. ELEKTROMOTORISCHES VERHALTEN VON SALZMISCHUNGEN.

Die rasche zeitliche Reversibilität, die man bei den beschriebenen P. D. Messungen beobachtet (Siehe S. 425), ist zweifellos durch die geringe Menge des in die nichtwässerige Phase übergegangenen Salzes bedingt; hierdurch ist es möglich, dass beim Aufbringen einer frischen Lösung sich das neue Gleichgewicht sofort einstellt. Diese rasche Reversibilität fehlt aber, wie zu erwarten, bei denjenigen (organischen) Salzlösungen, die nach der Theorie einen hohen Teilungskoeffizient besitzen. Denn bringt man einmal die organische Substanz mit einer solchen Salzlösung z. B. von Anilinhydrochlorid in Berührung, so sollte die dadurch entstehende hohe *Cl'*-Konzentration durch *NaCl*-Lösung nicht leicht wieder ausgewaschen werden. Dies ist auch tatsächlich der Fall; folgende Messungen mit *m*-Kresol als Mittelleiter sind ein Beispiel für solche Beobachtungen. (Apparat wie Fig. 1 auf S. 422.)

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[1]) Siehe J. Loeb u. R. Beutner, Biochem. Zeitschr. 59, 195 (1914).

Zeit in Minuten.	Lösung in Kontakt mit *m*-Kresol.	Spannung.
0'	*m*/10 *KCl*	+ 0.012 Volt
1	*m*/10 Dimethylanilin *HCl*	— 0.116 "
3	*m*/10 Dimethylanilin *HCl*	— 0.117 "

Darauf wurde das mit *m*-Kresol gefüllte U-Rohr sorgfältig mit destilliertem Wasser gewaschen, und die obere Schicht des Kresols zum Teil abgespült; wieder mit *m*/10 *KCl* in Berührung war trotzdem nach 6 Minuten die E. K. — **0.061 Volt.** Darauf wurde der Apparat ganz auseinander genommen, das untere U-Rohr mit Alkohol gespült und darauf wieder zusammengesetzt und mit frischem *m*-Kresol gefüllt, die E. K. bei Eintauchen des U-Rohrs im *m*/10 *KCl* war + 0.010 Volt. Darauf wurde gemessen:

mit *m*/10 *Na*-Oleat + 0.120 Volt (längere Zeit konstant);
wieder: *m*/10 *KCl* + **0.059 Volt.**

Beobachtungen dieser Art wurden mit allen möglichen andern Kombinationen wiederholt. Immer zeigte es sich, dass solche Lösungen, welche hohe negative oder hohe positive Potentiale hervorrufen, eine mangelhafte zeitliche Reversibilität zeigen, unter Versuchsbedingungen, bei denen sonst die Umkehrung momentan erfolgt. Zweifellos ist dies im Sinne der Theorie so zu deuten, dass das Salz mit dem grössern Teilungskoeffizienten (im obigen Beispiel Dimethylanilin *HCl*, resp. Natriumoleat) aus dem organischen Lösungsmittel nicht so leicht wieder vollkommen ausgewaschen werden kann.

Naturgemäss lassen diese Beobachtungen nicht leicht quantitative Messungen zu. Dies ist aber mit andern Versuchen ähnlicher Natur möglich: nämlich mit Messungen des elektromotorischen Verhaltens von binären Salzmischungen. Nach der Theorie ist zu erwarten, dass die P. D. sich nicht linear mit Zusammensetzung der Lösung (Verhältnis der molaren Mengen der beiden Salze) ändert.

Salze mit „organischer“ Säure oder Basis sollten in Mischung mit anorganischen Salzen die P. D. stärker beeinflussen, als nach ihrer relativen Menge zu erwarten ist. Hiermit stehen die Beobachtungen völlig in Übereinstimmung.

Messungen der Potentialdifferenz an der Phasengrenze von Acetophenon und wässerigen Lösungen, die $KSCN$ und K_2SO_4 in wechselndem Verhältnis bei konstanter Gesamtkonzentration ($m/10$) enthalten, ergaben folgendes:

$m/10\ K_2SO_4$	-0.034	Volt
$m/10\ (90\,^0/_0\ K_2SO_4 + 10\,^0/_0\ KSCN)$	$+0.096$	“
$m/10\ (80\,^0/_0\ K_2SO_4 + 20\,^0/_0\ KSCN)$	$+0.104$	“
$m/10\ (50\,^0/_0\ K_2SO_4 + 50\,^0/_0\ KSCN)$	$+0.124$	“
$m/10\ (20\,^0/_0\ K_2SO_4 + 80\,^0/_0\ KSCN)$	$+0.132$	“
$m/10\ (10\,^0/_0\ K_2SO_4 + 90\,^0/_0\ KSCN)$	$+0.133$	“
$m/10\ KSCN$	$+0.134$	“

Ein Zusatz von $10\,^0/_0$ $KSCN$-Lösung zu K_2SO_4-Lösung ändert die P. D. um 130 Millivolt, ein Zusatz von $10\,^0/_0$ K_2SO_4-Lösung zu $KSCN$-Lösung um 1 Millivolt. Die graphische Darstellung also gibt für die Änderung der P. D. eine gegen die Ordinatenachse stark konvexe Kurve (Fig. 2).

Mit Guajakol an Stelle von Acetophenon wurden ähnliche Versuche durchgeführt und das gleiche Verhalten beobachtet (die Ergebnisse sind in Fig. 3 graphisch wiedergegeben). Mit Mischungen von Trimethylamin HCl und $CaCl_2$ wurde ein analoges Verhalten beobachtet; da in diesem Falle das Salz, welches die negativere P. D. gibt, den grössern Teilungskoeffizient besitzt (gemeinsames Anion!), verläuft die Kurve konkav gegen die Ordinate (Fig. 4).

Diese Beobachtungen sind ein stichhaltiger Beweis für die Richtigkeit der hier entwickelten Vorstellungen[1]).

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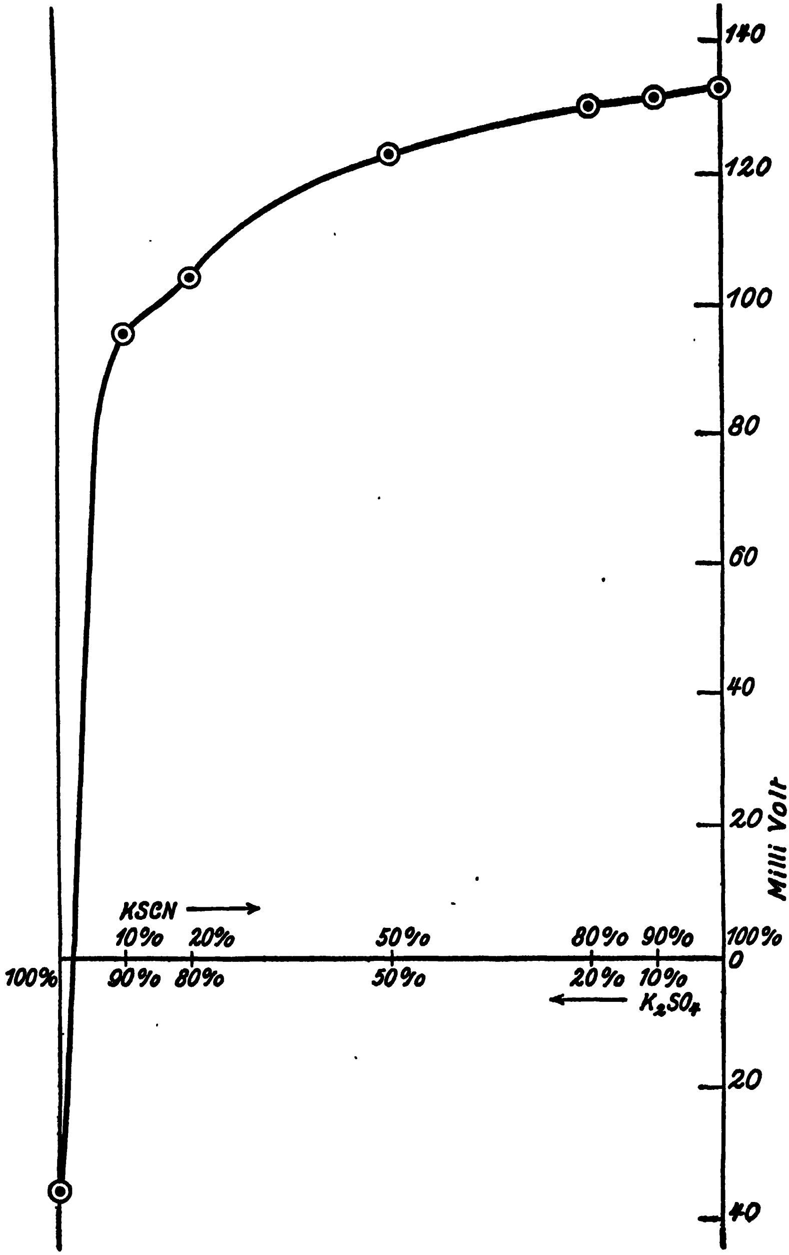
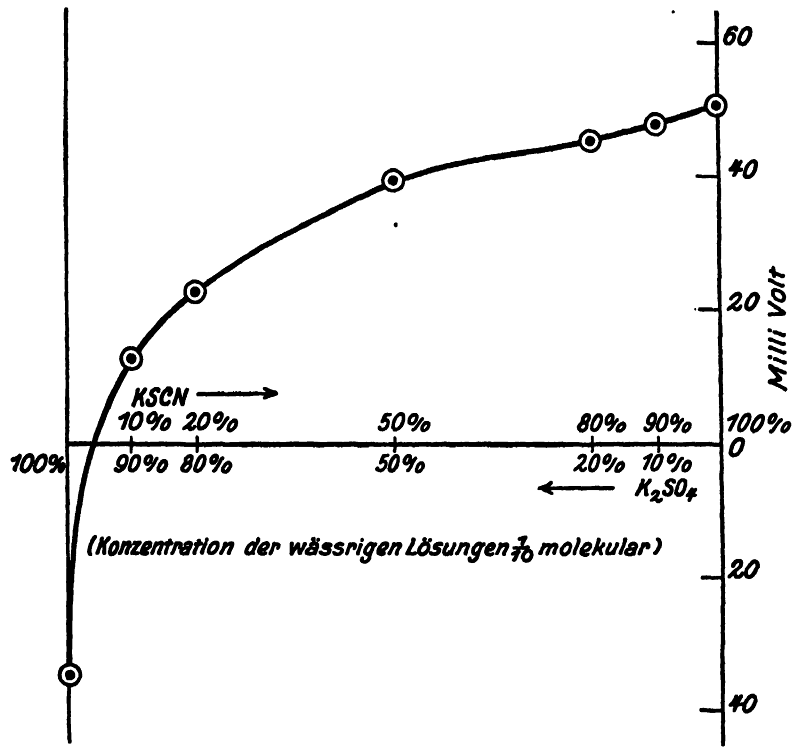
[1]) Das Verhalten der Potentialdifferenz gegen Salzmischungen in Lösung kann auch mit Hilfe der zuerst beschriebenen Theorie gedeutet werden. Da indes in einer frühern Arbeit des Verfassers gerade diese Verhältnisse ausführlich erörtert wurden, genügt hier der Hinweis.

Auf die Frage, ob die beiden hier benutzten Erklärungsmodi einander ausschliessen, oder ob sie beide gemeinsam beibehalten und nach Belieben angewendet werden können, muss die Antwort sicherlich im letztern Sinne lauten. Es



Änderung der Potentialdifferenz an der Phasengrenze von Acetophenon und wässerigen Lösungen, welche K_2SO_4 und $KSCN$ in wechselndem Verhältnis enthalten, so aber, dass die Gesamtkonzentration stets $m/10$ ist.

Fig. 2.



Änderung der Potentialdifferenz an der Phasengrenze von wässerigen $K_2SO_4 = KSCN$-Lösungen und Guajakol.

Fig. 3.

ist auch schon wohl bekannt, dass z. B. bei einer Kette, wie die von Bredig und Knüpffer untersuchten:

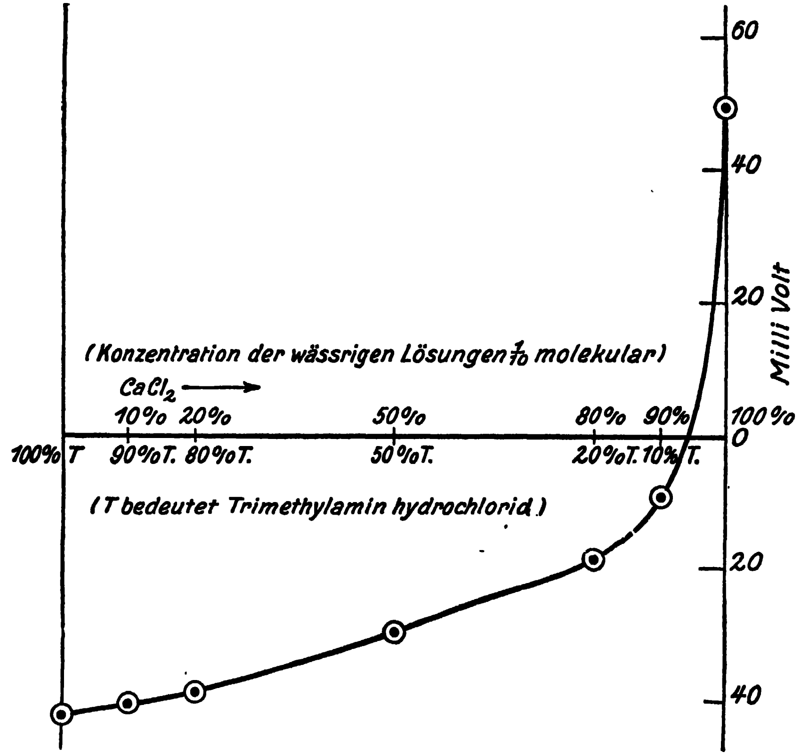
$$Tl \left| \begin{array}{cc} TlSCN & TlCl \\ \text{in } KSCN & \text{in } KCl \end{array} \right| Tl$$

die E. K. entweder auf Grund einer chemischen Reaktion, die bei Stromfluss vor sich geht, berechnet werden kann, oder nach der Nernstschen Formel als Konzentrationskette. Das letztere Verfahren, auf die hier beschriebenen neuartigen Ketten übertragen, führt eben zu der Berechnung der E. K. aus Teilungskoeffizienten. Da somit beide Berechnungsmethoden berechtigt sind, folgt weiter, dass zwischen der freien Energie chemischer Reaktion und Verteilungsgrössen sich durch thermodynamische Überlegungen ein Zusammenhang finden lässt. Auch dies ist nicht neu, da schon Bodländer die Löslichkeit von Silberhaloiden mit der Bildungswärme in Zusammenhang gebracht hat [Zeitschr. f. physik. Chemie 27, 55 (1898)]. Vor der Hand scheint es noch nicht lohnend, diese Beziehungen zu verfolgen.

7. UNTERSUCHUNGEN VON E. BAUR.

Adsorptionspotentiale.

Unter dem Titel: „Modell des elektrischen Organs der Fische", erschien kürzlich eine Mitteilung von E. Baur[1]), worin Ketten aus wässerigen Lösungen und wasserunmischbaren organischen Stoffen beschrieben sind, ähnlich den in derselben Zeitschrift kurz vorher vom Verfasser beschriebenen. Die Analogie derartiger Ketten mit den elektrizitäterregenden Systemen, die in lebenden Geweben vorliegen, war kurz vorher ebenfalls schon vom Verfasser angedeutet worden[2]). Baur beschreibt auch eine Anordnung, bestehend aus



Änderung der Potentialdifferenz an der Phasengrenze von wässerigem Trimethylaminhydrochlorid und $CaCl_2$-Lösungen und Guajakol.

Fig. 4.

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[1]) Z. f. Elektroch. 19, 590 (1913).

[2]) Loc. cit. siehe Z. f. Elektroch. 19, 475 (1913).

mehreren in geeigneter Weise hintereinander geschalteten Öl- und Wasserschichten (analog der bekannten Zambonischen Säule). Trotz der allgemeinen Ähnlichkeit dieser Ketten mit den in organischen Geweben vorliegenden elektrischen Systemen sind doch mit dem Bau eines so hoch differenzierten Organs wie des elektrischen, sicherlich viele physikalisch-chemischen Erscheinungen verknüpft, deren Natur uns bisher gänzlich unbekannt ist.

Die oben beschriebene Beobachtung, dass Alkalisalze organischer Säuren ein positives, Hydrochloride oder Sulfate organischer Basen ein negatives Potential geben, unabhängig von der chemischen Natur des Mittelleiters, bestätigt sich durch sehr bemerkenswerte Experimente E. Baurs in dieser Mitteilung. Die von ihm verwendete wasserunmischbare Substanz war ein kompliziertes Gemisch (im wesentlichen Ricinusöl und Tetrachloracetylen); in Kontakt z. B. auf der einen Seite mit K-Salicylat, auf der andern Seite mit Anilinhydrochlorid, wurde 0.1 Volt (zeitlich allerdings nicht konstant) gemessen. Die Richtung der E. K. war die gleiche wie bei den hier beschriebenen Messungen[1]). Mit einer Anzahl anderer Salzlösungen werden zum Teil recht hohe E. K. gemessen, stets in derselben Richtung. Da meine Messungen zeigen, dass für alle möglichen reinen Substanzen der „chemische“ Effekt der Salzlösungen in der gleichen Richtung liegt, ist es nicht erstaunlich, dass selbst komplizierte Gemische, wie Baur sie verwendet, die gleiche Wirkung hervorrufen.

Was nun die Erklärung dieser Wirkung betrifft, so werden von Baur die thermo-dynamischen Theorien der Phasengrenze P. D. und die experimentellen Untersuchungen von Haber u. a. (siehe oben) nicht berücksichtigt, sondern „Adsorptionspotentiale“ als Erklärungsprinzip genannt. Dieser Begriff ist zur Erklärung der koagulierenden Salzwirkung und endosmotischen Erscheinungen vielfach benutzt worden, obgleich er schwer zu begründen ist. Es darf wohl auch daran erinnert werden, dass Freundlich auf Grund neuerer Ergebnisse sich von dieser Theorie abgewandt hat, nachdem er sie früher befürwortet hatte[1]).

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[1]) Baur verwendet die wasserunmischbare Substanz nicht als Mittelleiter, sondern in direktem Kontakt mit dem ableitenden Metall. Seine Schaltung ist:

— *Hg* | Türkischrotöl | Kaliumsalicylatlösung | Anilinchlorhydratlösung |
Türkischrotöl | *Hg* +

Würde man das Türkischrot als Mittelleiter verwenden, analog wie bei den hier beschriebenen Versuchen die wasserunmischbare Substanz, so wäre die Natriumsalicylatlösung auf der positiven Seite, wie bei allen unsern Versuchen.

Nachdem in der vorliegenden Abhandlung gezeigt ist, dass sich solche elektromotorischen Phänomene auf Grund bewährter thermodynamischer Gesetzmässigkeiten (elektromotorische Phasengrenzregel, Verteilungssatz, Massenwirkungsgesetz) erklären lassen, muss eine Theorie, welche neue Vorstellungen einführt, sicherlich so lange zurückstehen, bis positive Beweise möglich sind.

8. ZUR THEORIE DER AGGLUTINATIONSERSCHEINUNGEN.

Schon mehrfach ist die Vermutung ausgesprochen worden, dass die Beständigkeit von Emulsionen durch die Existenz von Potentialdifferenzen (oder Ladungen) an der Oberfläche der Tröpfchen (oder festen Teilchen) bedingt ist. R. Ellis[2]) hat neuerdings hierfür eine einwandfreie experimentelle Bestätigung beigebracht. Er bestimmte die Ladung von Oltröpfchen in verschiedenen Salzlösungen aus der Wanderung derselben im elektrischen Feld (nach Helmholtz) und gleichzeitig die Geschwindigkeit, mit der dieselben sich zu grössern Massen zusammenballten (koagulierten). Es ergab sich, dass diese beiden Grössen völlig parallel gingen, und zwar war die Beständigkeit der Emulsion um so grösser, je grösser die Ladung der Teilchen war.

Sofern die Ladung oder Potentialdifferenz an der Phasengrenze kleiner Tröpfchen ähnlichen Gesetzmässigkeiten unterworfen ist[3]), wie an der Phasengrenze grosser Massen, können die hier gewonnenen Erklärungen auch auf solche durch elektrische Konvektion bestimmte Potentialdifferenzen übertragen werden. Die Messung der P. D. durch Endosmose ist andererseits der hier angewandten insofern überlegen, als sie absolute Werte der P. D. zu messen gestattet, während man durch Messungen von E. K. bekanntlich nur

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[1]) Zeitschr. f. physik. Chemie 79, 385 (1912).

[2]) Transact. of the Faraday Society 9, 14 (1913); Zeitschr. f. physik. Chemie 78, 321 (1911).

[3]) Diese Bedingung ist wahrscheinlich nur annähernd erfüllt, da auch die Grösse der Teilchen einen Einfluss haben soll (?).

Differenzwerte von P. D. bestimmen kann. Die in der Tabelle am Schluss der Arbeit mitgeteilten elektrometrischen Daten sind daher für die Theorie der Koagulationsphänomene nicht quantitativ zu verwerten. Andererseits ergibt sich aus der empirisch gefundenen Tatsache, dass der „chemische“ Effekt von Salzen auf die P. D. für alle Arten von wasserunmischbaren Stoffen qualitativ ähnlich ist, folgender Zusammenhang. Die koagulierende Wirkung von Alkalisalzen verschiedener Säuren ändert sich in folgender Anionenreihe: SCN', J', Br', Cl', NO_3', SO_4'', wie durch eine Anzahl von Untersuchungen gezeigt worden ist[1]. Es ist wohl kaum ein Zufall, dass, wie die Tabelle zeigt, auch für die Änderung der Potentialdifferenz an der Phasengrenze der meisten wasserunmischbaren Stoffe dieselbe Anionenreihe gültig ist. Nach den genannten Untersuchungen von Ellis ist es auch zu erwarten, dass ein solcher

							Nitrobenzol mit folgenden Zusätzen		
	Salicyl-aldehyd	*o*-Kresol	*m*-Kresol	*p*-Kresol	Phenol	*o*-Toluidin	1/5 g mol. *o*-Nitrobenzoesäure	1/5 g mol. Dimethylanilin	1/10 g mol. *o*-Nitrobenzoesäure + 1/10 g mol. Dimethylanilin
m/10 Na_2SO_4	− 3	− 42	− 25	− 36	−35	−120	+ 3	+26	+ 4
m/10 $NaCl$	0	0	0	0	0	0	0	0	0
m/10 $NaBr$	− 7	0	− 2	0	+ 1	+ 65	− 3	+20	+ 1
m/10 NaJ	0	+ 6	+ 4	+ 7	+ 8	+133	+ 3	+68	+ 8
m/10 $NaNO_3$	0	+ 10	+ 12	+ 10	+19	+ 67	− 3	+21	+ 3
m/10 $NaSCN$	+ 15	+ 25	+ 25	+ 25	+23	+140	+ 16	+92	+30
m/10 *Na*-Salicylat	+ 35	+ 60	+ 72	+ 60	+45	+120	−	−	−
m/10 *Na*-Benzoat	+ 35	+ 66	+ 56	+ 60	+45	+ 44	−	−	−
m/10 $NaOH$	−	+ 90	+140	+100	+52	+ 85	−	−	−
m/10 KCl	− 24	− 11	− 10	− 9	−12	0	− 49	0	+ 3
m/10 NH_4Cl	− 11	− 34	− 25	− 15	−14	− 1	− 24	− 3	+ 2
m/10 $BaCl_2$	+ 30	+ 25	+ 40	+ 34	+26	+ 7	+ 1	−	− 5
m/10 $CaCl_2$	+ 40	+ 30	+ 40	+ 37	+26	+ 10	+ 2	− 3	− 4
m/10 $MgCl_2$	+ 50	+ 20	+ 37	−	+29	+ 12	+ 8	− 2	− 4
m/10 HCl	− 10	− 54	− 3	− 4	− 5	−	− 57	−	−
m/10 Dimethylanilin HCl	−110	−138	−147	−116	−90	−	−210	− 6	−78
m/10 Dimethyltoluidin HCl	−124	−147	−	−	−	− 26	−	−	−
m/10 Anilin HCl	− 70	− 87	− 84	− 67	−39	−25[2]	−136	−	−40

[1] Die elektrometrische Kationenreihe ist ebenfalls mit der Koagulationswirkung verschiedener Salze mit gleichem Anion in Übereinstimmung.

[2] *m*/10 Toluidin HCl.

	Guajakol	Acetophenon	Benzylalkohol	Benzaldehyd	Anisaldehyd	Zimtaldehyd	Acetessigester
m/10 K_2SO_4	−34	− 35	−41	− 18	−32	+ 9	−32
m/10 KCl	−11	− 7	− 3	− 16	−12	− 12	−12
m/10 KBr	− 3	+ 32	+17	− 10	+12	+ 20	− 9
m/10 KNO_3	+14	+ 59	−	− 4	+29	+ 34	−
m/10 KJ	+20	+102	+48	+ 24	+63	+ 70	+25
m/10 $KSCN$	+47	+126	+55	+ 40	+82	+ 96	+40
m/10 $NaCl$	0	0	0	0	0	0	0
m/10 *Na*-Salicylat	+84	+107	+67	−	−	+ 75	+67
m/10 *Na*-Benzoat	+57	+ 40	+47	+ 50	−	+ 61	+14
m/10 *Na*-Oleat	+95	+109	+99	−	−	+ 69	−
m/10 $CaCl_2$	+55	+ 47	+15	+ 6	+ 5	+ 5	+42
m/10 $MgCl_2$	+45	+ 46	−	+ 9	+12	− 2	−
m/10 HCl	+26	−	− 6	− 28	−34	− 35	0
m/10 NH_4Cl	+ 2	−	+15	− 10	−13	−	− 6
m/10 Benzylamin HCl	−69	−	−37	− 84	−75	−	−67
m/10 Anilin HCl	−59	−	−35	− 74	−76	− 94	−45
m/10 Monomethylanilin HCl	−75	−	−42	− 86	−77	−108	−46
m/10 Dimethylanilin HCl	−91	− 74	−51	−100	−81	−	−52

Bemerkung zu den Tabellen.—Die im vorstehenden mitgeteilten Daten geben die relativen Werte der Potentialdifferenz in Millivolt, wobei die P. D. zwischen *m*/10 *NaCl*-Lösung und der betreffenden organischen Substanz willkürlich gleich Null gesetzt ist. Die Temperatur, bei der die Messungen ausgeführt wurden, war Zimmertemperatur (18—20°). Was die Reproduzierbarkeit derselben betrifft, so ist zu berücksichtigen, dass geringe Verunreinigungen der organischen Substanz erhebliche Effekte ausüben können. Die verwendeten Substanzen wurden vor dem Versuch durch Destillation gereinigt, ausser *o*-Toluidin; das reine *o*-Toluidin konnte nämlich wegen zu hohen Widerstands nicht zu elektrometrischen Messungen verwendet werden. Inwieweit Schwankungen der Temperatur die Genauigkeit beeinflussen, wurde bisher noch nicht untersucht, da jedenfalls innerhalb der Temperaturschwenkungen des Zimmers der Effekt nicht so gross ist, dass die wichtigsten Eigentümlichkeiten dieser neuen P. D., wie sie in der vorliegenden Abhandlung besprochen sind, dadurch verdeckt würden.

Zusammenhang existiert: Die Änderung der Ladung oder P. D. an der Phasengrenze der Teilchen verändert die Stabilität der Suspension oder kolloidalen Lösung. Da das Zustandekommen der Phasengrenzpotentialdifferenzen, wie wir gesehen haben, sich auf bekannte physikalisch-chemische Gesetzmässigkeiten zurückführen lässt, scheint es also auch möglich, für die komplizierten Koagulationswirkungen der Salze eine Erklärung zu finden. Weitere Untersuchungen müssen allerdings noch zeigen, inwieweit diese Erklärung wirklich zu Recht besteht.

ZUSAMMENFASSUNG.

Das wichtigste Ergebnis der Untersuchung ist folgendes: Durch zwei gleich konzentrierte wässerige Lösungen zweier Elektrolyte, welche durch eine Schicht einer wasserunmischbaren organischen Substanz (von elektrolytischem Leitvermögen) getrennt sind, wird eine E. K. erzeugt, die von den Teilungskoeffizienten dieser beiden Salze zwischen Wasser und der organischen Flüssigkeit gemäss der elektromotorischen Phasengrenzregel abhängt.

Folgende Beobachtungen werden als Stütze dieser Theorie beschrieben:

a) Bei Ketten, welche ein Hydrochlorid einer organischen Basis in der einen und *NaCl* in der andern Salzlösung enthalten, ist die erstere Lösung stets negativ. Dies ist im Sinne der Theorie, da man annehmen kann, dass für das Hydrochlorid der organischen Basis der Verteilungskoeffizient (organ. Lösungsmittel: Wasser) grösser ist als für *NaCl.* Bei Ketten, welche ein *Na*-Salz einer organischen Säure in der einen und *NaCl* in der andern Lösung enthalten, ist die erstere Lösung positiv. Dies entspricht dem grössern Teilungskoeffizienten für das organische *Na*-Salz.

b) Substanzen mit relativ hohen Verteilungskoeffizienten rufen eine zeitliche Irreversibilität der P. D. hervor.

c) In binären Salzlösungen ändert sich die E. K. nicht linear mit dem Mischungsverhältnis. Zusätze des Salzes mit dem grössern Teilungskoeffizienten beeinflussen, wie zu erwarten, die P. D. stärker wie gleiche Zusätze des Salzes mit den kleinern Teilungskoeffizienten.—

Da hiernach derartige Potentialdifferenzen durch thermodynamische Gesetzmässigkeiten zu erklären sind, ist die Einführung eines

Begriffs wie „Adsorptionspotential" (nach E. Baur) nicht zweckmässig.

Es wird ferner das Gesetz von der Unabhängigkeit der Ionenwirkung für einige derartige Potentialdifferenzen experimentell aufgefunden und thermodynamisch abgeleitet.

Es wird auf die Ähnlichkeit der elektromotorischen Ionenreihen mit der bei Agglutinationserscheinungen usw. beobachteten hingewiesen.

NACHTRAG: BERECHNUNG VON E. K. AUS LEITFÄHIGKEITSMESSUNGEN DER NICHT WÄSSERIGEN PHASE.

Nach Abschluss der vorliegenden Mitteilung gelang die Auffindung einer Methode der Bestimmung der Verteilung von Salzen zwischen organischer Flüssigkeit und Wasser, und damit für einige Fälle wenigstens die Berechnung der E. K. aus Beobachtungen anderer Art.

Durch Schütteln mit einer wässerigen Salzlösung erfährt nämlich die wasserunmischbare Substanz einen erheblichen Zuwachs an Leitfähigkeit infolge der geringen Salzmengen, die aus dem Wasser im Verteilungsgleichgewicht übertreten. Hiermit lässt sich eine Verteilung von Salzen selbst dann noch messen, wenn analytische Methoden, wie oben erwähnt, versagen. Eine Bestimmung von wahren Verteilungskoeffizienten ist natürlich nicht möglich, da man weder über Ionenbeweglichkeit, noch über den Grad der elektrolytischen Dissociation in den wasserunmischbaren Lösungsmitteln sicheres weiss. Nur die relative Ionenkonzentration in dem wasserunmischbaren Lösungsmittel lässt sich so schätzen. Dies ist aber gerade die für die Theorie wichtige Grösse; dieselbe sollte dem Logarithmus der Änderung der E. K. proportional sein. Folgende Messungen mit Guajakol zeigen in der Tat eine Proportionalität zwischen dem Logarithmus der Zunahme der Leitfähigkeit beim Schütteln und der E. K. So z. B. wurde Guajakol von einer Eigenleitfähigkeit von 0.1 reziproken Megohm mit folgender $^1/_{10}$-molekularen Lösung von Chloriden geschüttelt (15 ccm Guajakol + 50 ccm wässerige Lösung), die nach dem Schütteln beobachtete Zunahme der Leitfähigkeit (K) wurde der Cl-Konzentration proportional gesetzt und die Änderung der E. K. infolgedessen berechnet als $\frac{RT}{F} \ln \frac{K_1}{K_2}$ konst. Folgende Tabelle zeigt die Übereinstimmung.

Leitfähigkeit des Guajakols nach Schütteln mit einer $^1/_{10}$-mol. Lösung des folgenden Salzes bei Zimmertemperatur[1]).		Berechnete relative E. K.	Beobachtete E. K.
Dimethylanilin HCl	59.0 rez. Megohm.	— 100 Millivolt	— 91 Millivolt
Anilin HCl	10.3 " "	— 56 "	— 59 "
KCl	1.9 " "	— 12 "	— 11 "
$NaCl$	1.2 " "	± 0 (willkürl.)	± 0 "
$MgCl_2$	0.3 " "	+ 43 "	+ 45 "

Trotz geringer Abweichungen durch Versuchsfehler zeigen diese Zahlen deutlich, wie in einer Serie von Salzen mit gemeinsamem Anion ein negativeres Potential mit einer grössern Leitfähigkeit des Guajakols parallel geht, infolge des stärkern Eindringens des Salzes.

Bei einem Vergleich verschiedener Salze derselben Basis, z. B. K_2SO_4, KCl, KNO_3, KJ, $KSCN$, ergab sich zwar auch ein Parallelismus zwischen Leitfähigkeit der Guajakolschicht E. K., jedoch keine logarithmische Proportionalität, also nur eine qualitative, keine quantitative Übereinstimmung. Die Ursache dieser Abweichung bleibt aufzuklären, vermutlich spielen Verteilungserscheinungen, wie sie auf S. 429 erörtert wurden, eine störende Rolle. Das betreffende Salz geht nicht nur als solches vom Wasser in das Guajakol über, sondern es bilden sich auch Guajakolsalzverbindungen. Eine andere Erklärungsmöglichkeit könnte man durch Annahme von Diffusionspotentialen im Guajakol finden. Eine Prüfung dieser Hypothesen bleibt weitern Untersuchungen vorbehalten.

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[1]) Messung der Leitfähigkeit bei $t = 30°$.

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CLUSTER FORMATION OF SPERMATOZOA CAUSED BY SPECIFIC SUBSTANCES FROM EGGS.

BY JACQUES LOEB.

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INTRODUCTION.

In several papers F. Lillie[1] has described a very interesting specific phenomenon of apparent sperm agglutination which occurs when the sperm is mixed with sea water which has been in contact for a short time with a sufficient quantity of eggs of the same species (Arbacia and Nereis):

> In the case of *Arbacia* the addition of two or three drops of egg-sea water ¼ (i. e., one volume of eggs to four volumes of sea water) which has stood half an hour, to about 2 cc. of fresh milky sperm suspension causes formation of agglutinations 1 to 2 mm. in diameter in a few seconds. The agglutination may be so strong that the fluid between the white agglutinated masses appears perfectly clear. The masses gradually fade from view in a few minutes, but microscopic agglutinations may remain half an hour or more.

The agglutination is, therefore, only transitory or reversible, as Lillie states. It is specific since e.g., the supernatant sea water of Arbacia eggs act only on Arbacia sperm and not on other sperm.

It is very natural that Lillie should have been led to the idea that such a striking specific phenomenon as this agglutination must play a rôle in the process of fertilization and he has recently offered a very carefully worked out hypothesis which makes this phenomenon of agglutination not only the center of the process of fertilization and of artificial parthenogenesis but he even hints that it may be involved in the phenomena of heredity.

Lillie's theory of the phenomenon of agglutination is an application of Ehrlich's side-chain theory, a fact which gives it additional interest.

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[1] Science, N.S., vol. 36, p. 527, 1912; Journ. Exper. Zoöl., vol. 14, p. 515, 1913.

> In previous papers I have described the secretion of a substance by the ova of the sea urchin, *Arbacia,* in sea water, which causes agglutination of the sperm of the same species. The eggs of *Nereis* also secrete a substance having a similar effect upon its sperm. I therefore named these substances sperm-isoagglutinins. During the present summer I have ascertained that in the case of *Arbacia,* and presumably also of *Nereis,* the agglutinating substance is a necessary link in the fertilization process and that it acts in the manner of an amboceptor, having one side-chain for certain receptors in the sperm and another for certain receptors in the egg. As this substance represents, presumably, a new class of substances, analogous in some respects to cytolysins, and as the term agglutinin defines only its action on sperm suspensions, I have decided to name it fertilizin.[2]

The writer had for many years observed that when the eggs of the Californian sea urchin, Strongylocentrotus purpuratus, were fertilized with sperm of their own species the spermatozoa would not always scatter but would form small clusters which were often visible with the naked eye. These clusters would disappear in a few minutes. The whole phenomenon resembles strikingly the phenomenon described by Lillie under the name of sperm agglutination, and is possibly identical with it.

The writer was interested to find out what the conditions of this cluster formation of the sperm and its relation to the process of fertilization were. Since he is not certain whether this cluster formation observed by him on the Californian sea urchin is identical with the observations of Lillie on the agglutination of sperm in Arbacia, he will confine himself to a discussion of his own experiments and observations, leaving it for future work to decide to what extent they harmonize with Lillie's observations and conclusions.

METHOD OF OBSERVATION AND THE SPECIFIC CHARACTER OF CLUSTER FORMATION.

If we put one or more drops of a very thick sperm suspension of the Californian sea urchin, S. purpuratus, carefully into the center of a dish containing 3 cc. of ordinary sea water, and let the drops stand for one-half to one minute, and then by gentle agitation mix the sperm with the sea water, the at first rather viscous mass of thick sperm is in a few seconds distributed equally in sea water and the result is a homogeneous sperm suspension.

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[2] Science, N.S., vol. 38, no. 980, p. 524, October 10, 1913.

When the same experiment is made with the sea water which has been standing for a short time in a dish over a large mass of eggs of the same species, the result is entirely different. The thick drop of sperm seems to be less miscible and instead of a homogeneous suspension of sperm we get as a result the formation of a large number of distinct clusters which are visible to the naked eye and may possess a diameter of 1 or even 2 mm. The rest of the sea water is almost free from sperm. These clusters of spermatozoa last for from two to ten minutes and then dissolve by the gradual detachment of the spermatozoa from the periphery of the clusters. This phenomenon is to some extent specific. The sperm of the sea urchin Strongylocentrotus purpuratus will give the cluster formation with the supernatant sea water of the eggs of S. purpuratus; the sperm of the sea urchin S. franciscanus will give the cluster formation with the supernatant sea water of eggs of its own kind as well as with the supernatant sea water of the eggs of S. purpuratus. In the latter case the clusters dissolve a little more quickly than if franciscanus sperm is added to the supernatant sea water of franciscanus eggs. The sperm of purpuratus will not form clusters with the supernatant sea water of the eggs of franciscanus. It is of interest that the specificity is not reciprocal in the case of these two sea urchins.

The sperm of neither formed clusters with the supernatant water of starfish eggs or of mollusc eggs.

The sperm of starfish (Asterias ochracea and Asterina) gave no cluster formation with the supernatant sea water of their own eggs or of the eggs of the two sea urchins.

We shall have to return to these data in a later chapter when we discuss the relation between cluster formation and fertilization.

The following experiments were carried on with the sperm of S. purpuratus and the supernatant sea water of the eggs of the same species, unless the contrary is stated.

APPARENT SURFACE TENSION PHENOMENA AND CLUSTER FORMATION.

In analyzing the formation of these clusters the writer was struck with the fact that the cluster formation showed peculiarities which resembled the action of surface tension. The clusters were usually spherical, or had a tendency to become so. When two clusters were brought into contact with each other they fused at once into one spherical cluster with a larger radius, a behavior which would also be observed in the case of drops of substances immiscible with water under similar conditions. The formation of the clusters themselves resembled surface tension phenomena. When a drop of purpuratus sperm is gently agitated in a little dish with a few cubic centimeters of ordinary sea water streaks and cylindrical masses of sperm are formed in the water which, however, show nothing that reminds one of surface tension phenomena. The spermatozoa are gradually scattered without surface tension offering any resistance to the scattering.

If the same experiment is made in the supernatant sea water from the eggs—in egg-sea water—the streaks of sperm produced by agitation behave somewhat like cylinders of a very viscous substance which is immiscible with water, e.g., a viscous oil or a calcium soap. Short streaks or cylinders contract into spherical masses, the above described clusters; and long cylinders break up into a series of small clusters.

In an attempt to account for this apparent or real rôle of surface tension in cluster formation the writer thought first of the possibility that it might be due to an agglutination of the masses of sperm under the influence of the egg-sea water. A study of the real phenomenon of sperm agglutination, however, showed that it does not lead to any formation of spherical clusters. The writer had shown eleven years ago that real sperm agglutination can be produced if we add 2 or 3 cc. of $\frac{N}{10}$ NaOH to 50 cc. of sea water.[8] He found recently a good method of producing sperm agglutination with less alkali in the case of the sperm of starfish. When this sperm is put into 50 cc. sea water + 0.5 cc. $\frac{N}{10}$ NaOH it shows a tendency to agglutinate only after about one hour. But we can produce a real agglutination of the spermatozoa after about only twenty minutes when we put the sperm into the supernatant sea water of eggs or sperm of purpuratus. This agglutination is not specific, since it can also be produced by a great many other substances, e.g., cattle serum or even white of egg. In this case the spermatozoa at first stick together to form short rows or threads; and later the threads begin to stick together and form irregular networks. At no time is there any appearance of cluster formation or anything suggesting the phenomena of surface tension.

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[8] Loeb, Arch. f. d. ges. Physiol., Bd. 99, p. 323, 1903; Bd. 104, p. 325, 1904.

The writer is therefore under the impression that the cluster formation of the sperm in the supernatant sea water of its own eggs is a phenomenon of a different type from agglutination by alkali.

MOTILITY OF SPERM AND CLUSTER FORMATION.

In observing the clusters the writer was struck with the fact that the spermatozoa at the periphery of a cluster are in free progressive motion, a fact which is incompatible with the assumption of agglutination. When the clusters were small or when the sperm suspension was thin it was possible to observe the spermatozoa which are in the center of the cluster. It was seen that the spermatozoa in the center also were in very lively motion, with the possible exception of small lumps or groups of spermatozoa which may have stuck together. The clusters reminded the writer of a dense swarm of insects which move like a coherent mass through space. These clusters move like one solid body through the water, notwithstanding the fact that the individual spermatozoa are free to scatter.

Under the influence of these observations the writer formed the idea that the cluster formation and possibly the apparent phenomena of surface tension might be the outcome of some tropistic reaction of the spermatozoa. If this were the case, we should expect that anything that diminished the motility of the spermatozoa would lessen the tendency of the sperm to form clusters, and if the sperm were paralyzed completely the cluster formation would also cease completely.

It was easy to show that both assumptions were correct. To 3 cc. of a dense sperm suspension in ordinary sea water were added 1 or 2 drops of a 0.1 per cent solution of NaCN, and the whole thoroughly mixed. In one or two minutes the sperm lost its motility and did not regain it when put into sea water. When one or several drops of this immobilized sperm were added to the egg-sea water and when after one minute the dish was gently agitated, the sperm behaved exactly as if it had been put into normal sea water. Not a trace of cluster formation was noticeable; a slight agitation sufficed to bring about a perfectly homogeneous mixture of the sperm in sea water. After two hours the sperm became motile again when put into sea water. When such sperm, after the recovery of its motility, was put into egg-sea water a very powerful cluster formation occurred again.

These experiments were varied and always proved definitely that the whole phenomenon of cluster formation existed only when the sperm was motile.

There are other ways of paralyzing the spermatozoa. When the sperm of purpuratus is heated to a temperature of 35°C. or even 36°C. the sperm remains motile and the phenomenon of cluster formation is striking when a drop of such sperm is added to 3 cc. of egg-sea water and the mass is agitated. As soon as sperm is brought to a temperature of 37.6° or above and rapidly cooled, the motility is gone and no cluster formation takes place.

The same experiment was made with the sperm of Strongylocentrotus franciscanus and the supernatant sea water of eggs of the same species. When the sperm is heated to a temperature of 36.2° its motility continues and the cluster formation is not diminished. When the sperm is heated for one minute to a temperature of 37° the motility of the sperm is only diminished and only small clusters are formed. If the sperm is heated to 38° the motility of the sperm disappears and the phenomenon of cluster formation is impossible.

The same result is obtained if the motility of the sperm is diminished or annihilated through the addition of KCl to sea water.

These are all very striking demonstration experiments, which leave no doubt that the cluster formation and the apparent surface tension phenomena depend exclusively on the motility of the spermatozoa.

On the other hand, the writer convinced himself that in the true phenomena of sperm agglutination, the motility of the sperm is of no concern. We have mentioned the fact that the sperm of Asterias, when it has been in 50 cc. sea water + 0.5 cc. $\frac{N}{10}$ NaOH for fifteen or twenty minutes, undergoes a real agglutination when mixed with the supernatant sea water of different kinds of eggs or of cattle serum. This real agglutination takes place just as well after the spermatozoa have been completely immobilized by KCN as before.

THE CONDITIONS WHICH DETERMINE THE SCATTERING OF THE CLUSTER.

The clusters (just like Lillie's 'agglutinations') have only a short duration of from two to ten minutes, as the circumstances may be. It was of interest to find out some of the conditions which determine their duration. It was found that the stability of the clusters depends upon the alkalinity of the sea water. In a neutral solution the big clusters may last a considerable time, half an hour or more, while in sea water to which a sufficient amount of alkali has been added the clusters may scatter in a minute. The reader must remember that if we add HCl or NaOH to sea water part of the added acid or base will be neutralized by the carbonates and phosphates of the sea water.

To 5 cc. supernatant sea water from purpuratus eggs were added 0, 1, 2, 3, 4 drops of $\frac{N}{10}$ NaOH and 3 drops of a dense suspension of purpuratus sperm were added to each. In all dishes a large cluster was formed. In the dishes with 4 and 3 drops of NaOH the clusters were dissolved almost instantly after formation, in the dish with 2 drops the resolution occurred more slowly and it lasted longest—about eight minutes—in the sea water to which no alkali was added.

In a second experiment to 5 cc. of the same egg-sea water 0, 1, 2, 3 and 4 drops $\frac{N}{10}$ HCl were added, and then purpuratus sperm introduced. In 5 cc. egg-sea water + 4 drops of HCl no cluster formation occurred, probably because the motility of the sperm was too rapidly annihilated. In the dish with 3 drops HCl only a trace of cluster formation was noticeable; with 2 drops a moderate cluster formation occurred and only in the two dishes with 0 and 1 drop of HCl was the cluster formation very powerful, since here the motility of the spermatozoa was not impaired. In the egg-sea water without acid the clusters disappeared much more quickly than in the sea water with 1 or 2 drops of acid.

Experiments in which neutral artificial sea water was substituted for normal sea water showed that at the point of neutrality the cluster formation is most durable. The big clusters continued to exist as long as half an hour, while in alkaline solutions they disappeared very rapidly. In acid solutions no cluster formation was possible probably on account of the fact that the spermatozoa became immobile.

These and other experiments prove that an increase in the alkalinity of the solution shortens the duration of the clusters, in spite of the fact that an increase in alkalinity of the sea water favors the real agglutination of sperm.

The writer tried then to ascertain which salt solutions favor the formation of these clusters. To investigate this point the ovaries and testes of purpuratus were washed in an m/2 NaCl solution and then put directly into another m/2 NaCl solution without coming in contact with sea water. It was found that the supernatant solution of the eggs did never, or only exceptionally, give rise to cluster formation with the NaCl sea water; the reason for this may be partly the fact that the spermatozoa are practically inactive in a pure NaCl solution and that although the presence of the supernatant NaCl solution from the eggs stimulates the spermatozoa into activity this may not always be sufficient. The addition of KCl does not materially improve the cluster formation, the addition of the chlorides of Mg, Ca, Sr and Ba and of $MgSO_4$ vastly increases the cluster formation or induces it in an otherwise inefficient NaCl solution.

It is not possible to draw any conclusions from these facts upon the nature of the process underlying it.

THE ORIGIN OF THE SUBSTANCE CAUSING THE CLUSTER FORMATION.

Lillie assumes that the substance which causes the phenomenon described by him as agglutination is given off by the egg itself, though he states that the jelly which surrounds the egg—viz., the chorion—is saturated with this substance. The writer was curious to know whether the phenomenon of cluster formation depends upon a substance given off by the egg or whether it is due to a substance originating from the chorion. It could easily be shown that the latter is the case. Herbst had stated that the chorion of the sea urchin egg can be dissolved by acid. The writer therefore put a mass of eggs of purpuratus for three minutes into 50 cc. sea water + 3 cc. $\frac{N}{10}$ HCl. The eggs were constantly squirted with a pipette to prevent them from sticking to the glass and were then transferred to normal sea water. They were then washed five times in succession in normal sea water under constant squirting with a pipette and then left standing in a refrigerator with a small volume of sea water. At no time did the sea water in which these eggs were kept give any trace of a cluster formation with fresh sperm. The supernatant sea water was tested a few hours after the acid treatment and two or three times daily on four consecutive days. These eggs which had apparently lost their chorion had permanently lost the power of giving off to the sea water a substance which causes the cluster formation of the spermatozoa of the same species. If the substance were constantly given off by the egg it should have been found after some time in the supernatant sea water. The experiment was repeated a number of times with the same negative result.

On the other hand, it was easy to show that the acid sea water (50 cc. sea water + 3 cc. $\frac{N}{10}$ HCl) in which the eggs had been washed contained the substance which is responsible for the cluster formation in large quantities. This acid sea water was filtered and the filtrate neutralized with NaOH (with neutral red as an indicator). The neutralized sea water gave with sperm of the same species a very powerful cluster formation.

This neutralized sea water kept the power of inducing cluster formation for about three days (during which time it stood in the refrigerator) but had lost it the fourth day.

This seems to indicate that the substance causing cluster formation is derived from the jelly-like substance surrounding the egg (the chorion) but does not emanate from the egg itself.

If this substance which causes the cluster formation should be identical with the substance which Lillie calls 'fertilizin,' which is very probable, it is obvious that his conclusion that the substance comes from the egg is untenable for the egg of purpuratus. This would also make it impossible to attribute to this substance a rôle in the process of artificial parthenogenesis.

CLUSTER FORMATION AND FERTILIZATION.

Lillie's application of the side-chain theory to the problem of fertilization rests on the assumption that the substance which causes the phenomenon he describes as agglutination is indispensable for fertilization. This 'fertilizin' is in his theory an amboceptor which must combine at one end with the spermatozoön, at another end with the egg; the 'fertilizin' when in combination with the spermatozoön undergoes a change and then fertilizes the egg. It is therefore a substance given off by the egg which in Lillie's opinion causes its fertilization, and not, as we all had hitherto assumed, one or more substances contained in the spermatozoön.

We have just seen that the substance which causes the cluster formation does not come from the egg of purpuratus but is given off by the chorion—or possibly is the chorion itself which is slowly soluble in sea water.

We can show in a number of different ways that eggs which have lost or do not possess the power of giving off a substance which induces cluster formation may possess the normal power of being fertilized. If we treat eggs of purpuratus for three minutes in 50 cc. sea water + 3 cc. $\frac{N}{10}$ HCl and wash them about five times in sea water they have lost the power of causing the cluster formation of the sperm of purpuratus. Such eggs can be fertilized immediately after the washing or at any time during the next two or three days if they are kept in the refrigerator. Their power of being fertilized is not in the least impaired. One hundred per cent of the eggs were invariably fertilized and the fertilization took place instantly after the addition of sperm. Practically all the eggs developed. The membrane was slightly abnormal which was an after-effect of the acid treatment. The power of the eggs of being fertilized remains unimpaired while their power of giving off substances which cause cluster formation is completely and permanently lost.

When we treat the eggs with a fatty acid instead of with a mineral acid they form, when transferred to normal sea water, a fertilization membrane. The fatty acid also dissolves the chorion and such eggs when washed afterwards lose their power of inducing cluster formation of sperm. It is interesting that Lillie states that such eggs lost also their power of causing agglutination, which seems to suggest that Lillie's 'agglutination' and the writer's 'cluster formation' may be the same thing. Lillie states that eggs which have formed a fertilization membrane have lost their power of being fertilized and he sees in this a support of his theory that without 'fertilizin' the egg can no longer be fertilized.

Eggs which have formed a fertilization membrane under the influence of butyric acid can easily be fertilized with sperm if the membrane is torn by shaking. The writer has repeated this experiment this winter and confirmed the earlier observations by Kupelwieser and himself to the same effect. He also made sure that the eggs which after the artificial membrane formation can be fertilized with sperm have completely lost the power of causing cluster formation.

We thus see that complete loss of the power of inducing cluster formation can be combined with maximal power of the eggs of being fertilized.

HYBRIDIZATION AND CLUSTER FORMATION.

The best test for a possible connection between fertilization and cluster formation is afforded in the phenomena of hybridization. If the phenomenon of cluster formation were inseparably associated with the power of the eggs of being fertilized, we should expect that sperm should only be able to fertilize the eggs of a species if the egg-sea water of the same species caused the cluster formation of the sperm.

It is easy to show that no connection of this type exists. It is impossible to call forth cluster formation of the spermatozoa of the starfish Asterias ochracea with the egg-sea water of purpuratus and yet 100 per cent of the eggs of purpuratus can be fertilized with the sperm of ochracea and as many as 80 per cent of these eggs may develop. The writer showed that this hybridization takes place only in hyperalkaline sea water and it was therefore necessary to test the possibility of cluster formation in both neutral and alkaline sea water; these tests gave always absolutely negative results.

The sperm of purpuratus shows no trace of cluster formation with the egg-sea water of franciscanus and yet the eggs of franciscanus are readily fertilized with the sperm of purpuratus.

If the cluster formation were caused by a substance which was necessary for fertilization in the sense of Lillie's theory these and probably many other hybridizations which occur should be impossible.

It often happens that in hybridization less than 100 per cent of the eggs are fertilized. The writer tried whether the yield of fertilized eggs could be increased if the egg-sea water from the species which furnishes the sperm be added to the mixture. This would furnish the sperm with the specific 'amboceptor.' It was found that hybridizations occur just as well if not better in normal sea water than if the egg-sea water from the species from which the sperm is taken be added.

All these facts contradict the assumption that the substance which induces the cluster formation of the spermatozoön is necessary for fertilization.

ARTIFICIAL PARTHENOGENESIS AND CLUSTER FORMATION.

The writer has expressed the idea that the causation of the development of the egg either by a spermatozoön or by the agencies of artificial parthenogenesis is due to an alteration of the cortical layer of the egg which may or may not be accompanied by a membrane formation; and he has shown that all hemolytic substances are able to bring about this alteration. Lillie agrees with this idea but differs in regard to the origin of the agent which causes this change in the cortical layer of the egg in the case of fertilization by a spermatozoön. According to the writer, this change is caused by a substance contained in the spermatozoön while Lillie assumes that it is a substance contained in the egg which must, however, be activated by the spermatozoön in order to cause the alteration of the surface of the egg. It seems to the writer that Lillie's assumption is unnecessarily complicated. Moreover, if it should turn out that the substance which is responsible for the cluster formation is identical with the substance which Lillie calls 'fertilizin'—which is very likely the case—Lillie's theory becomes untenable, since this substance does not, in all probability, originate from the egg but from the chorion and since there is, as we have seen, no connection between the presence of this substance and the power of the eggs of being fertilized.

A second difficulty which Lillie has not considered lies in the fact that the writer has shown that in addition to the membrane forming substance still another, namely a corrective agency, is necessary for the causation of the development of the egg. The cortical change induces development but the egg as a rule perishes if the second factor of artificial parthenogenesis is not applied (hypertonic solution or lack of oxygen). The writer is suspicious that even a third factor may be implied. It is under these circumstances difficult to see how the assumption that the 'fertilizin' causes development—leaving aside all other objections—can act as an adequate substitute for the known facts of artificial parthenogenesis.

Lillie sees a proof for his idea in the statement of Glaser[4] that the filtrate from eggs of Arbacia ground up with an equal volume of sea water will cause normal unfertilized eggs of the same species to undergo one or more cell divisions if they are transferred from the filtrate after one or two hours to normal sea water. Lillie concludes from this that the egg contains its own fertilizing substance, the 'fertilizin,' and that it was this 'fertilizin,' liberated from the eggs when they were ground up and contained in the filtrate, which induced the cell division of the Arbacia eggs in Glaser's experiments. Waiving the question of how this '' fertilizin' was 'activated' without the presence of sperm, the writer sees no reason to assume that the egg extract acted through the 'fertlizin,' or any other specific substance, since he has shown that a large number of non-specific substances are able to induce the first cell divisions (without membrane formation) in the egg of Arbacia, e.g., traces of any weak base like HN_3, or protamine.[5] A slight increase of osmotic pressure could also have such an effect. It even suffices to let the eggs of certain females lie for some time in sea water.[6] The writer fails to see any reason for assuming that the cell division in Glaser's experiments was induced by 'fertilizin.' The fact that the composition of the mixture which he used was unknown is not sufficient proof that 'fertilizin' was the active agency.

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[4] Science, N.S., vol. 38, no. 978, p. 446, 1913.

[5] Jour. Exp. Zoöl., vol. 13, p. 577, 1912; Arch. f. Entwcklngsmechn. d. Organ., Bd. 38, p. 409, 1914.

In conclusion, the writer would like to call attention to the fact that the cluster formation described in this paper or the agglutination of sperm described by Lillie inhibit the fertilizing effect of the spermatozoa instead of enhancing it, since the cluster formation prevents the spermatozoa from reaching the egg. Even from a teleological viewpoint it is difficult to understand why a substance which only prevents the fertilizing action of the sperm should be a necessary link in such action. The writer is inclined to believe that the cluster formation or agglutination of sperm does not occur when fertilization takes place under natural conditions.

The writer is not quite sure whether his interpretation of the cluster formation of the spermatozoa as a tropistic reaction will have to be modified or not. If it should turn out to be essentially or partly correct, it might be worth while to point out that we have many examples of tropistic reactions which are of no possible use to the species, e.g., all the phenomena of galvanotropism which are only laboratory products.

The writer takes pleasure in expressing his thanks to Prof. S. S. Maxwell of the University of California for his kindness in putting the Herzstein Laboratory at New Monterey at the writer's disposal.

SUMMARY.

1. The writer describes the formation of clusters of spermatozoa which is observed when the sperm of a sea urchin is put into the supernatant sea water of eggs of the same species. This specific phenomenon of cluster formation may possibly be identical with the specific phenomenon described under the name of sperm agglutination in recent publications of F. Lillie.

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[6] Arch. f. Entwcklngsmechn. d. Organ., Bd. 36, p. 626, 1913.

2. The cluster formation resembles the phenomena of surface tension in various respects, e.g., inasmuch as the clusters are spherical or tend to assume a spherical shape, and inasmuch as the fusion of two clusters results in the formation of a larger spherical cluster. When sperm is put into ordinary sea water or the supernatant water of foreign eggs these apparent surface tension phenomena are not observed. In real sperm agglutination neither cluster formation nor the above-mentioned surface tension phenomena are noticeable.

3. It was found that the cluster formation is a direct function of the motility of the spermatozoa. As soon as the spermatozoa are immobilized by NaCN or by high temperature or by KCl the cluster formation ceases; as soon as the motility of the spermatozoa returns the cluster formation occurs again when the sperm is put into the supernatant sea water of eggs of the same species. The real agglutination of sperm occurs just as well when the sperm is immobilized as when it is motile. The cluster formation is therefore not a form of agglutination.

4. The clusters last only a few minutes, like Lillie's sperm 'agglutinations.' The writer has found that in a neutral solution they last much longer than in an alkaline solution and that they scatter the more rapidly the more the alkalinity of the sea water is raised by the addition of NaOH.

5. It is shown that eggs which have been treated with acid sea water lose permanently their power of producing a substance which causes the cluster formation of the spermatozoa of their own species; while the acid sea water in which the eggs were treated when filtered and neutralized with NaOH induces a very powerful cluster formation. If it is true that the acid dissolves the chorion (the jelly-like substance surrounding the egg) this experiment would prove that the substance which causes the cluster formation is not formed in the egg but in the chorion. If the substance is identical with the substance which Lillie calls 'fertilizin,' his theory concerning the rôle which this substance plays in the fertilization and development of the egg will meet with serious difficulties.

6. It is shown that eggs which have been treated with a mineral acid like HCl and which have permanently lost the power of caus-

ing a cluster formation of the spermatozoa can nevertheless all be fertilized with sperm of the same species and that the rapidity with which the sperm fertilizes these eggs is equal to that with which normal eggs are fertilized. When the acid used was a fatty acid and when membrane formation occurred the eggs also lost permanently their power of inducing cluster formation but retained their power of being fertilized by sperm, provided that the membrane was first torn.

7. The supernatant sea water of the eggs of Strongylocentrotus franciscanus will not induce cluster formation of the sperm of Strongylocentrotus purpuratus; yet the latter sperm fertilizes the eggs of franciscanus. The sperm of Asterias ochracea undergoes no cluster formation in the supernatant sea water of Strongylocentrotus purpuratus, no matter whether the sea water is normal or hyperalkaline although the starfish sperm readily fertilizes most or all the eggs of Strongylocentrotus purpuratus in hyperalkaline sea water.

8. The facts mentioned under paragraphs 6 and 7 prove that the substance which is responsible for the cluster formation is not necessary for the process of fertilization.

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DER IRREZIPROKE CHARAKTER DES ANTAGONISMUS ZWISCHEN ANIONEN UND KATIONEN.*

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I.

In seinen frühesten Versuchen über die antagonistischen Salzwirkungen am Fundulusei hatte der Verf. gezeigt, dass bei diesen Erscheinungen die elektrische Ladung und die Wertigkeit der Ionen eine in mancher Hinsicht ähnliche Rolle spielen wie bei der Fällung von kolloidalen Lösungen[1]). Es war nämlich möglich, die Giftwirkung von Salzen mit einwertigem Kation durch Spuren von Salzen mit einem zweiwertigen Kation aufzuheben, während die Salze mit mehrwertigem Anion keine entgiftende Wirkung hatten. Es ergab sich bei diesen Versuchen u. a. das paradoxe Resultat, dass die Giftwirkung einer Chlornatriumlösung durch Zusatz so giftiger Salze wie die von Barium, Zink, Mangan u. a. verringert werden konnte. Bei diesen Versuchen handelte es sich um eine Wirkung der Ionen auf die Durchgängigkeit der Membran, welche die Fischeier umgibt. Diese Membran ist für Salze und Wasser praktisch undurchgängig. In einer reinen NaCl-Lösung von der Konzentration, in der dieses Salz im Seewasser enthalten ist, wird die Membran rasch durchgängig und das führt zum Tode des Embryo. Der Zusatz einer Spur eines Salzes mit zweiwertigem Kation verhindert diese Erhöhung der Durchgängigkeit[2]). Es liegt nahe anzunehmen, dass die Wirkung der mehrwertigen Kationen in einer Fällung eines Kolloids besteht, wie das zuerst von T. B. Robertson vermutet wurde.

Während diese Versuche an den Eiern der Fische ausgeführt

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* *Eingegangen am 10. Juni 1914.*

[1]) Loeb, Arch. f. d. ges. Physiol. 88, 68, 1901; Amer. Journ. of Physiol. 6, 411, 1902; Loeb und Gies, Arch. f. d. ges. Physiol. 93, 246, 1902.

[2]) Loeb, diese Zeitschr. 47, 127, 1912.

waren, sind die folgenden Versuche an den ausgewachsenen Fischen angestellt.

Wir wollen in dieser Abhandlung auf die Irreziprozität im Antagonismus von Kationen und Anionen hinweisen, nämlich, dass es leicht gelingt Anionen durch mehrwertige Kationen zu entgiften, dass aber die Entgiftung von Kationen durch mehrwertige Anionen nicht oder nur in geringem Masse gelingt. Die Tatsachen, von denen schon in den früheren Arbeiten gelegentlich die Rede war, sind von der einfachsten Art. Wenn wir Lösungen verschiedener Natriumsalze in den Konzentrationen herstellen, in denen sie die Fische (Fundulus) in weniger als einem Tage töten, so gelingt es mittels des Zusatzes von $CaCl_2$ oder $MgCl_2$ oder anderer Salze mit zweiwertigem Kation, die Fische eine Reihe von Tagen am Leben zu halten[1]). Machen wir nun den umgekehrten Versuch und stellen wir die ebenso giftige Konzentration eines Salzes her, bei dem das Kation das nachweisbar oder vermutlich giftige Salz ist (z. B. $CaCl_2$, $MgCl_2$, LiCl, NH_4Cl), so gelingt es gar nicht oder fast gar nicht, eine merkliche Entgiftung durch Zusatz mehrwertiger Anionen herbeizuführen. So sterben die Fische in Natriumcitratlösungen, deren Konzentrationen über $^m/_{400}$ liegt in weniger als einem Tage; fügt man der Lösung aber etwas $CaCl_2$ oder $MgCl_2$ zu, so leben sie eine lange Reihe von Tagen. Das Anion des Calciumsalzes ist dabei von untergeordneter Bedeutung. In den folgenden Versuchen ist die Lebensdauer der Fische in Tagen angegeben. Je 4 Fische waren in 500 ccm der Lösung. Die Lebensdauer ist die Zeit, in der der letzte Fisch starb. 0 bedeutet, dass die Fische in weniger als 24 Stunden alle starben.

Der Versuch (Tabelle I) ist typisch für viele ähnliche, die angestellt wurden. Während die Fische schon in einer $^m/_{400}$ bis $^m/_{200}$ Natriumcitratlösung alle in weniger als 24 Stunden starben, leben sie in einer 8 oder 16 mal stärkeren Lösung von Natriumcitrat 12 bis 14 Tage, wenn man nur etwas $CaCl_2$ oder $MgCl_2$ zusetzt. $CaCl_2$ ist in $^m/_{100}$-Lösungen wirksamer als $MgCl_2$. In $^m/_{100}$-$CaCl_2$ bewirken die Lösungen von Natriumcitrat, die hier benutzt wurden, keinen Niederschlag.

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[1]) Loeb, diese Zeitschr. 32, 308, 1911.

TABELLE I.

Giftige Lösung Natriumcitrat. Entgiftende Lösung $MgCl_2$ resp. $CaCl_2$.
Lebensdauer der Fische (in Tagen).

Entgiftende Lösung	$\frac{m}{200}$	$\frac{m}{100}$	$\frac{2m}{100}$	$\frac{3m}{100}$	$\frac{4m}{100}$	$\frac{6m}{100}$	$\frac{8m}{100}$
	Natriumcitrat						
0 (Kontrolle)	0	0	0	0	0	0	0
$\frac{m}{100}$ $MgCl_2$	14	14	0	0	0	0	0
$\frac{m}{16}$ $MgCl_2$	3	16	15	14	15	4	0
$\frac{m}{8}$ $MgCl_2$	10	6	12	13	13	13	14
$\frac{m}{4}$ $MgCl_2$	2	3	2	2	2	3	2
$\frac{m}{8}$ $MgSO_4$	3	4	4	3	3	1	0
$\frac{m}{100}$ $CaCl_2$	16	5	14	12	12	0	0

$CaCl_2$, $CaBr_2$ und $CaSO_4$ wirkten ungefähr gleich gut.

Giftige Lösungen von Na_2SO_4, weinsaures, bernsteinsaures, essigsaures und salpetersaures Natrium werden in ebenso schlagender Weise durch geringe Mengen von $MgCl_2$ resp. $CaCl_2$ entgiftet. Die minimale, für die Entgiftung ausreichende Konzentration von $MgCl_2$ ist stets etwas höher als die von $CaCl_2$.

Wir können demnach die allgemeine Regel aufstellen, dass Natriumsalze, in der Konzentration, in der sie anfangen giftig zu wirken, durch die Salze mit zweiwertigem Kation (besonders Mg und Ca) entgiftet werden können. Die Calciumsalze wirken in etwas geringerer Konzentration entgiftend als die Magnesiumsalze.

II.

Wir führen nun den umgekehrten Versuch aus, nämlich sehen zu, ob die giftigen Konzentrationen von Salzen, bei denen das Kation der giftige Bestandteil ist, durch Salze mit mehrwertigem Anion entgiftet werden können. Das ist praktisch nicht der Fall.

Hier müssen wir eine kurze Betrachtung darüber einschieben, dass in einer giftigen $MgCl_2$-Lösung das Mg-Ion und nicht das Cl-Ion die Giftigkeit bestimmt. Das folgt aus folgender Überlegung. In einer $^{m}/_{2}$-NaCl-Lösung lebt Fundulus meist mehrere Tage, und das Leben wird beliebig verlängert, wenn wir etwas $MgCl_2$ oder $CaCl_2$ hinzufügen. Hier könnte das Cl-Ion das giftige Ion sein, da ja das Mg-Ion entgiftend wirkt. In einer $^{m}/_{4}$-$MgCl_2$-Lösung sterben die Fische aber in weniger als 24 Stunden. Hierfür kann also nur das Mg-Ion verantwortlich sein, da ja die giftige Wirkung der Cl-Ionen durch den Überschuss von Mg-Ionen aufgehoben ist.

Es wurde nun der Versuch gemacht, ob es gelingt die giftige Wirkung einer $^{m}/_{4}$-$MgCl_2$-Lösung durch Zusatz eines Salzes mit mehrwertigem Anion zu verringern. $^{m}/_{4}$-Lösungen von $MgCl_2$ wurden in destilliertem Wasser sowie in $^{m}/_{50}$-, $^{m}/_{25}$-, $^{3}/_{50}$-, $^{2\,m}/_{25}$-, $^{m}/_{10}$-, $^{6\,m}/_{50}$-, $^{15\,m}/_{100}$-Lösungen von Natriumtartrat,-succinat, -sulfat und -citrat hergestellt. Im besten Falle war es möglich, die Lebensdauer der Fische um einige Stunden zu vermehren, aber nicht um viele Tage, wie das im umgekehrten Versuche der Fall war. Es findet also nur eine minimale Entgiftung der Magnesiumsalze durch die Salze mit zwei- oder mehrwertigem Anion statt.

Mit Calciumsalzen lassen sich derartige Versuche wegen der Niederschlagsbildungen entweder nicht oder nur in beschränktem Masse anstellen. Ebensowenig werden die Salze von Kalium, Lithium und Ammonium durch SO_4-Ionen entgiftet. Alle Versuche, Salzlösungen, in denen die Giftwirkung nachweisbar oder möglicherweise durch ein Kation bedingt war, durch Salze mit mehrwertigen Anionen zu entgiften, schlugen fehl. Alle früheren Versuche des Verfassers stützen diesen Schluss.

III.

Wir dürfen nun die Frage aufwerfen, ob auch die beiden entgegengesetzten Ionen desselben Salzes antagonistisch aufeinander wirken können. Es liegt auf der Hand, dass der Nachweis einer solchen Tatsache für das Verständnis der biologischen Salzwirkungen von grosser Bedeutung sein würde. Bei der Erörterung dieser Frage müssen wir aber eine Tatsache erwähnen, die für die Beurteilung der antagonistischen Salzwirkungen sehr wesentlich ist. Wir können, wie gesagt, eine Natriumchlorid- oder Natriumsulfat- oder Natriumcitratlösung, welche die Grenze der Giftigkeit erreicht, leicht durch Zusatz von Spuren von $MgCl_2$ oder $CaCl_2$ entgiften. Fügen wir aber wachsende Mengen von $MgCl_2$ oder (wo es sich um lösliche Calciumverbindungen handelt) $CaCl_2$ zu, so erreichen wir schliesslich eine Grenze, bei der der weitere Zusatz des $MgCl_2$ oder $CaCl_2$ die Giftigkeit der Lösung wieder erhöht. Das tritt ein, wenn die Konzentration der $MgCl_2$- oder $CaCl_2$-Lösung die Grenze erreicht, bei der das Mg oder Ca seine giftige Wirkung zu entfalten beginnt.

Nun besitzen die verschiedenen Salze eines Ions sehr verschiedene Grade der Giftigkeit; bestimmen wir beispielsweise die minimale Konzentration, bei der die verschiedenen Natriumsalze die Fische in weniger als einem Tage töten, so finden wir sehr grosse Unterschiede, die wir nur auf die Verschiedenheiten der Wirksamkeit des Anions beziehen können. So ist diese giftige Konzentration für NaCl etwas über $^5/_8$ m, für Na_2SO_4 etwas über $^m/_8$ und für Natriumcitrat $^m/_{400}$. Die folgende Tabelle zeigt das etwas ausführlicher.

TABELLE II.

Natur des Salzes	Minimum der giftigen Konzentration
NaCl	etwa $^5/_8$ m
NaBr	etwa $^m/_5$
NaJ	$^m/_{20}$
NaCNS	$^m/_{200}$
$NaCH_3COO$	$^m/_5$
$NaNO_3$	$^m/_{20}$
Na_2SO_4	etwa $^m/_8$
Weinsaures Na	$^m/_{25}$
Bernsteinsaures Na	$^m/_{20}$
Oxalsaures Na	$^m/_{166}$
Citronensaures Na	$^m/_{400}$

Wenn nun tatsächlich eine gegenseitige Entgiftung der entgegengesetzt geladenen Ionen eines Salzes stattfindet, so muss sich das darin zeigen, dass im Falle eines sehr giftigen Anions die Natriumsalze giftiger sein müssen als die betreffenden Magnesium-, Calcium- oder Strontiumsalze¹). Das ist auch allgemein der Fall. Tabelle III gibt die minimalen Konzentrationen von $NaNO_3$, $Mg(NO_3)_2$,

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¹) Weil ja die früheren Versuche des Verfassers gezeigt haben, dass Spuren der Salze mit zweiwertigem Metall die Salze mit einwertigem Metall entgiften, dass aber das gleiche nicht in demselben Grade durch Salze mit einwertigem Metall möglich ist.

$Ca(NO_3)_2$, $Sr(NO_3)_2$ und $Ba(NO_3)_2$, welche die Fische in weniger als einem Tage töten.

TABELLE III.

Natur des Salzes	Minimum der giftigen Konzentration
$NaNO_3$	$^m/_{20}$
$Mg(NO_3)_2$	$^m/_{10}$
$Ca(NO_3)_2$	$^m/_8$
$Sr(NO_3)_2$	$^m/_{10}$
$Ba(NO_3)_2$	$^m/_{50}$
$LiNO_3$	$^m/_{50}$
NH_4NO_3	$^m/_{50}$

Während also eine $NaNO_3$-Lösung schon in einer Konzentration von $^m/_{20}$ die Fische in weniger als einem Tage tötet, hat eine $Mg(NO_3)_2$-Lösung diese Wirkung erst bei einer Konzentration, die 2 mal so hoch ist. $NaNO_3$ ist also 2 mal so giftig wie $Mg(NO_3)_2$, 2 $^1/_2$ mal so giftig wie $Ca(NO_3)_2$ und 2 mal so giftig wie $Sr(NO_3)_2$. Nur $BaCl_2$ macht eine scheinbare Ausnahme, die aber darin ihre Erklärung findet, dass Bariumsalze sich nur zur Entgiftung bei Fischeiern eignen, wo sie nicht mit den lebenden Tieren in direkte Berührung kommen; dass sie aber bei dem erwachsenen Fische nur eine sehr geringe entgiftende Wirkung üben.

Das Gesagte gilt ganz allgemein für Salze, deren Anion sehr giftig ist. Der Verfasser hat schon früher gezeigt, dass $CaBr_2$, essigsaures Calcium und $Ca(OH)_2$ viel weniger giftig sind als die entsprechenden Natriumsalze[1]). Auch die Magnesiumsalze dieser Anionen sind weniger giftig als die Natriumsalze. In $^m/_5$ Na_2SO_4 leben die Fische nur wenige Stunden, in $^m/_5$ $MgSO_4$ leben sie eine Woche oder länger. In einer $^m/_{10}$-NaBr-Lösung lebten die Fische nur 2 Tage, in einer $^m/_{10}$-$MgBr_2$-Lösung lebten sie 5 Tage.

Diese Tatsachen stützen die Annahme, dass die beiden entgegengesetzten Ionen eines Salzes eine antagonistische Wirkung aufeinander ausüben, was dann besonders deutlich zutage tritt, wenn das Anion relativ giftig ist. Handelt es sich aber um ein relativ ungiftiges Anion wie Cl, das bereits durch Na hinreichend entgiftet wird, so wird die Giftigkeit von $MgCl_2$, $CaCl_2$ und $SrCl_2$, wie wir sehen, durch das Kation bestimmt. In dem Falle ist NaCl weniger giftig als $MgCl_2$ oder $CaCl_2$.

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[1]) Diese Zeitschr. 39, 194, 1912.

Die zweite Folgerung aus dem Gedanken, dass die Ionen desselben Salzes eine antagonistische Wirkung aufeinander ausüben, würde die sein, dass in den Fällen, in denen das Kation die Giftigkeit bestimmt, die Salze mit mehrwertigem Anion fast ebenso giftig sind wie die Chloride. Das folgt aus der Tatsache, dass die Giftigkeit der Salze mit mehrwertigem Kation nur wenig bzw. gar nicht herabgesetzt wird. Eine Lösung von $MgCl_2$ tötet die Fische in einer $^m/_5$-Lösung meist in 1 oder 2 Tagen. Wie der Vergleich mit der Giftigkeit von NaCl ergibt, muss das Mg-Ion die obere Giftigkeitsgrenze von $MgCl_2$ bestimmen. Der Zusatz von etwas Na_2SO_4 verringert die Giftigkeit von $MgCl_2$, aber nur in geringem Grade. Wir finden nun, dass $MgSO_4$ etwas weniger giftig für die Fische ist als $MgCl_2$. So leben die Fische in einer $^m/_5$-$MgSO_4$-Lösung ungefähr 1 Woche, während sie in einer $^m/_5$-$MgCl_2$-Lösung nur 1 bis 2 Tage leben. Es ist aber nicht ausgeschlossen, dass hier der Umstand mit hereinspielt, dass wegen des höheren Grades der elektrolytischen Dissoziation die Konzentration der Magnesiumionen in der $MgCl_2$-Lösung höher ist als in der $MgSO_4$-Lösung derselben Konzentration.

$LiCl_2$, KCl und NH_4Cl sind sehr giftig für die Fische, und die geringe Giftigkeit des Chlorions sowie die Tatsache, dass beispielsweise KCl sich für diese Tiere durch NaCl entgiften lässt, weist darauf hin, dass das Kation in diesen Salzen das giftige Ion ist. Es war von Interesse, zu untersuchen, ob die Salze dieser drei Kationen mit mehrwertigen Anionen weniger giftig sind als ihre Chloride. Das ist aber nicht der Fall, wie Versuche mit den Chloriden und Sulfaten von Kalium, Lithium und Ammonium zeigten.

Wir kommen daher zum Schlusse, dass auch die Ionen desselben Salzes sich gegenseitig antagonisieren und dass die Kationen die Anionen kräftiger zu antagonisieren imstande sind als umgekehrt. Wir müssen also die Frage aufwerfen: Was bedingt diesen irreziproken Charakter des Antagonismus? Ehe wir diese Frage beantworten können, müssen wir einige Betrachtungen über die Theorie der antagonistischen Salzwirkung vorausschicken.

IV.

Die Idee, dass ein Antagonismus zwischen den Ionen entgegengesetzter Ladung besteht, scheint im Gegensatz zu der früher schon vom Verfasser mitgeteilten Beobachtung zu stehen, dass die Giftwirkung von Anionen nicht nur durch mehrwertige Kationen, sondern auch durch ein bestimmtes Anion, nämlich Cl, verringert werden kann. So konnte der Verfasser zeigen, dass die Giftwirkung von NaBr, NaJ, Na_2SO_4, essigsaurem Natrium und anderen durch Zusatz von NaCl aufgehoben oder erheblich verringert werden kann[1]). Ebenso hatte er schon vorher den Nachweis geliefert, dass auch die Giftwirkung von KCl bei Fundulus nicht nur durch $CaCl_2$, sondern auch durch NaCl aufgehoben bzw. verringert werden kann[2]). Hier handelt es sich um Entgiftung durch gleichsinnige Ionen.

Wie soll man es nun verstehen, dass beide Erscheinungen möglich sind, nämlich 1. ein Antagonismus von Ionen mit entgegengesetzter Ladung und 2. ein Antagonismus von Ionen von gleicher Ladung? Der Verfasser vermutet, dass die eigentliche Variable bei den antagonistischen Salzlösungen ein bestimmter Zustand der Kolloide der Oberfläche des Fisches ist.

Versuche, die noch nicht veröffentlicht sind, haben ergeben, dass die normale Haut von Fundulus für Wasser sowohl wie für Salze undurchgängig ist. Variiert man die Konzentration des umgebenden Seewassers innerhalb gewisser Grenzen, so ändert sich die Undurchgängigkeit der Membran nicht. Wohl aber ist das der Fall, wenn man diese Grenze überschreitet[3]). Noch leichter wird diese Änderung herbeigeführt durch Lösungen, die nur ein einziges Salz enthalten. Wir nehmen an, dass die Undurchgängigkeit der Membran daher rührt, dass bestimmte Ionen (nämlich in diesem Falle die des Seewassers) mit gewissen Kolloiden der Haut chemische Verbindungen eingehen und dass der physikalische Charakter dieser Verbindungen die Eigenschaft der Membran bestimmt. Ersetzt man Seewasser durch die Lösung nur eines einzigen Salzes, so ändert sich die Konstitution der Membran und damit ihre Durchgängigkeit. Anionen und Kationen beeinflussen die Membran in entgegengesetztem Sinne, aber wenn der Überschuss von Calciumionen eine gewisse Grenze überschreitet, so zerstört er die Membran ebenso sicher, wie ein Überschuss von beispielsweise Citrationen. Wir sollten nun auf Grund dieser Annahme erwarten, dass eine Mischung von $NaCl + KCl + CaCl_2$ in dem Verhältnis, in dem diese Salze im Seewasser enthalten sind, eine starke antagonistische Wirkung gegen die meisten Salze ausübt. Das trifft auch zu. In einer früheren Arbeit habe ich bereits gezeigt, dass das für Salze mit einwertigen Anionen der Fall ist, wie beispielsweise NaBr, NaJ, $NaNO_3$, essigsaures Natrium u. a.[1]). Es trifft aber auch für Salze wie KCl, LiCl und NH_4Cl zu, in denen das giftige Ion sehr wahrscheinlich das Kation ist. Solche Salze können nun genau wie die Salze von $NaNO_3$ oder NaBr durch künstliches Seewasser kräftig entgiftet werden. Die folgenden drei Tabellen zeigen die entgiftende Wirkung von künstlichem Seewasser in verschiedener Konzentration, sowie von $CaCl_2$- und NaCl-Lösung. Unter künstlichem Seewasser ist hier die Mischung von $NaCl + KCl + CaCl_2$ verstanden.

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[1]) Loeb, diese Zeitschr. 43, 181, 1912.

[2]) Diese Zeitschr. 31, 450, 1911.

[3]) Diese Zeitschr. 53, 391, 1913.

TABELLE IV.

Giftige Lösung LiCl. Entgiftende Lösung künstliches Seewasser. Lebensdauer in Tagen.

Entgiftende Lösung	$\frac{2m}{25}$	$\frac{m}{10}$	$\frac{3m}{25}$	$\frac{3m}{20}$	$\frac{7m}{40}$	$\frac{m}{5}$ LiCl
0 (Kontrolle).................	1	1	0	0	0	0
$\frac{m}{100}$ künstliches Seewasser......	4	2	0	1	0	0
$\frac{m}{16}$ " "	4	5	2	3	3	1
$\frac{m}{8}$ " "	5	3	3	1	1	1
$\frac{m}{4}$ " "	4	2	2	1	1	1
$\frac{3m}{8}$ " "	2	2	1	0	0	0

Die entgiftende Wirkung des künstlichen Seewassers ist sehr deutlich. NaCl-Lösungen hatten keine so deutliche entgiftende Wirkungen, wie Tabelle V zeigt.

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[1]) Diese Zeitschr. 43, 181, 1912.

TABELLE V.

Giftige Lösung LiCl. Entgiftende Lösung NaCl.
Lebensdauer in Tagen.

Entgiftende Lösung	$\frac{2m}{25}$	$\frac{m}{10}$	$\frac{3m}{25}$	$\frac{3m}{20}$	$\frac{7m}{40}$	$\frac{m}{5}$ LiCl
0 (Kontrolle)	0	0	0	0	0	0
$\frac{m}{100}$ NaCl	0	0	0	0	0	0
$\frac{m}{16}$ NaCl	6	2	0	0	0	0
$\frac{m}{8}$ NaCl	4	1	0	0	0	0
$\frac{m}{4}$ NaCl	1	1	1	0	0	0
$\frac{3m}{}$ NaCl	1	0	0	—	—	—

Calciumchlorid wirkt dagegen ebenso stark entgiftend wie das künstliche Seewasser.

TABELLE VI.

Giftige Lösung LiCl. Entgiftende Lösung $CaCl_2$.
Lebensdauer in Tagen.

Entgiftende Lösung	$\frac{2m}{25}$	$\frac{m}{10}$	$\frac{3m}{25}$	$\frac{3m}{20}$	$\frac{7m}{40}$	$\frac{m}{5}$ LiCl
0 (Kontrolle)	0	0	0	0	0	0
$\frac{m}{100}$ $CaCl_2$	3	3	3	2	3	2
$\frac{m}{16}$ $CaCl_2$	3	3	3	3	2	2
$\frac{m}{8}$ $CaCl_2$	3	2	0	2	1	1
$\frac{m}{4}$ $CaCl_2$	0	0	0	0	0	0

Ähnlich wie $CaCl_2$ wirken auch $MgCl_2$ und $MgSO_4$.

Die Versuche mit NH_4Cl zeigen ebenfalls, dass künstliches Seewasser besser entgiftet, als die Lösung irgendeines anderen Salzes. Hier liegt aber die Komplikation vor, dass die NH_4Cl-Lösungen schwach sauer sind, was es wohl bedingt, dass auch NaCl eine bessere entgiftende Wirkung gegen NH_4Cl hat als gegen LiCl.

Wir sehen also, dass künstliches Seewasser (und zweifellos auch natürliches Seewasser) eine noch viel stärkere entgiftende Wirkung hat als irgendein einzelnes Salz oder Ion. Nur wenn das giftige Salz ein Salz mit mehrwertigem Anion ist, wie Natriumcitrat, mag es vorkommen, dass das Seewasser nicht ganz so günstig wirkt wie Mg, aber der Unterschied ist nicht sehr gross.

Alle diese Tatsachen lassen sich unter der Annahme verstehen, dass die Salze den Zustand gewisser Kolloide der Haut des Fisches bestimmen. Eine Änderung im einen wie im entgegengesetzten Sinne schädigt die Haut, sobald die Änderung eine gewisse Grösse überschreitet. Der Umstand, dass entgegengesetzt geladene Ionen die Kolloide im entgegengesetzten Sinne beeinflussen, erklärt den Antagonismus zwischen Anionen und Kationen. Der Umstand, dass das Optimum der Undurchlässigkeit der Haut dann vorhanden ist, wenn die Fische im normalen Milieu, d. h. in Seewasser sind, lässt sich unter der Annahme verstehen, dass die Salze der umgebenden Lösung mit den Kolloiden an der Oberfläche der Fische chemische Verbindungen eingehen (oder dass sie dieselben in anderer Weise beeinflussen) und dass der spezifische Charakter dieser Verbindungen die physikalische Natur und Eigenschaften der Haut oder ihrer Oberfläche bestimmt. Auf Grund dieser Annahme muss der Ersatz des Seewassers durch eine andere Lösung zu einer chemischen Änderung der Haut und damit zu einer Änderung ihrer physikalischen Eigenschaften, insbesondere zu einer Erhöhung der Durchlässigkeit führen. Für die Erhaltung dieser normalen physikalischen Beschaffenheit ist offenbar irgendeine Wirkung von Ca und Mg auf die Kolloide von besonderer Bedeutung. Nehmen wir an, dass diese Wirkung praktisch irreversibel ist (beispielsweise eine Fällung), während die Wirkung der polyvalenten Anionen reversibel ist, so verstehen wir auch den irreziproken Charakter der antagonistischen Wirkung von Anionen und Kationen, von dem hier die Rede ist.

V. ZUSAMMENFASSUNG DER RESULTATE.

1. Es wird gezeigt, dass die Wirkung giftiger Anionen, organischer sowohl wie anorganischer, durch Spuren von zweiwertigen Kationen wie Mg und Ca aufgehoben werden kann.

2. Es wird ferner gezeigt, dass die giftige Wirkung, die Kationen (z. B. Mg, Ca, K, Li, NH_4) in genügend hoher Konzentration ausüben, durch mehrwertige Anionen gar nicht oder nur in verschwindendem Masse verringert werden kann.

3. Es wird damit bewiesen, dass der Antagonismus zwischen Anionen und Kationen irreziprok ist.

4. Es wird gezeigt, dass derselbe irreziproke Antagonismus zwischen den beiden Ionen desselben Salzes besteht, indem beispielsweise die Magnesiumsalze oder Calciumsalze sehr giftiger Anionen (wie z. B. NO_3, Br, SO_4, Essigsäure u. a.) bei weitem nicht so giftig sind wie die Natriumsalze derselben Anionen; während beispielsweise die Sulfate giftiger Kationen (wie z. B. K, Li u. a.) gar nicht oder nur wenig besser sind als die Chloride.

5. Es wird eine Theorie dieser Erscheinungen entwickelt.

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ON SOME NON-SPECIFIC FACTORS FOR THE ENTRANCE OF THE SPERMATOZOON INTO THE EGG.

BY JACQUES LOEB, M.D.

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1. While formerly fertilization was considered a single process which could be adequately described by the entrance of the spermatozoon into the egg or the fusion of the egg nucleus with the sperm nucleus, we know now, through the methods of experimental biology, that fertilization consists of at least three different groups of phenomena. These are, first, the transmission of paternal characters through the spermatozoon. This process is obviously a function of the chromosomes. Second, the causation of development of the egg, which is apparently independent of the chromosomes since the experiments on artificial parthenogenesis have shown that it can be induced by certain non-specific agencies. The causation of development is a complicated process since it requires at least two agencies, one inducing an alteration of the surface of the egg (which sets the chemical processes underlying development in action), and the other a corrective agency which guarantees a normal development.

The third group of factors involved in the process of fertilization is that determining the entrance of the spermatozoon into the egg. This note will deal with the latter problem.

2. We can undertake the analysis of the conditions necessary for the entrance of the spermatozoon into the egg from two different starting points, namely, by finding means for fertilizing the eggs with the sperm of distant species against which the egg is naturally immune; or by rendering the eggs immune against sperm of their own species. The former problem was solved for certain cases when the writer found that the eggs of the sea urchin (*Strongylocentrotus purpuratus*) which under normal conditions can not be fertilized by the sperm of the starfish or holothurians can be fertilized with such sperm if the sea-water is rendered more alkaline.

Last winter the writer found that an addition of calcium chloride to sea-water acts in the same way. In this case the above-mentioned hybridization can be brought about if little or no alkali is added to the sea-water. This suggested the idea that the forces determining the entrance of the spermatozoon into the egg depended upon the concentration of calcium and hydroxylions in the sea-water.

3. If this idea was correct it was to be expected that the elimination of these two substances might render the eggs which are naturally fertilized in normal sea-water immune against sperm of their own species. This was found to be the case. If eggs and sperm of *Arbacia* or *purpuratus* are freed from sea-water and put into a neutral mixture of $NaCl + KCl$ or $NaCl + MgCl_2$, or of $NaCl + KCl + MgCl_2$ (in the concentration and proportion in which these salts exist in the sea-water) no egg is fertilized. Yet it can be seen that sperm remains motile in these solutions for a long time (twenty-four hours or longer) and it can also be shown that newly fertilized eggs are able to segment in these solutions. If calcium chloride is added to these solutions fertilization will take place at once. The same is true when a trace of a base is added to the mixture of $NaCl + MgCl_2$ or of $NaCl + KCl + MgCl_2$.

On the other hand, these eggs can be fertilized by sperm of their own species in neutral solutions containing calcium, namely $NaCl + CaCl_2$ or $NaCl + KCl + CaCl_2$, or $NaCl + CaCl_2 + MgCl_2$, or $NaCl + KCl + MgCl_2 + CaCl_2$. Similar results were obtained in regard to the fertilization of the eggs of an annelid (*Chœtopterus*) and a mollusk (*Cumingia*). It can, therefore, be stated that the entrance of a spermatozoon into an egg of its own or foreign species is determined by forces which are influenced by the concentration of calcium and hydroxylions in the solution, the difference in both cases being only in the concentration of these substances required.

4. The question arises, which forces in the egg or spermatozoon are influenced by these two agencies. Since it seems tolerably certain that neither the strong base nor the calcium salts enter into the egg or the spermatozoon, the forces acted upon by these substances must be located at the surface of the egg or spermatozoon. There are only three kinds of forces that need be taken into consideration;

namely, (1) surface tension, (2) adhesion between spermatozoon and egg surface, (3) cohesion or degree of fluidity of the surface of the egg. Experiments which the writer carried out last winter in Pacific Grove seem to indicate that the adhesion of the spermatozoon to other bodies is strongly influenced by both calcium and bases. The egg of the sea urchin is surrounded by a jelly which the spermatozoon must penetrate before it reaches the egg. If it should stick to the inner surface of the jelly it might still come in contact with the egg and might be able to impart to the surface of the egg the membrane-forming substance; but through its adhesion to the jelly it might be prevented from entering the egg. The egg should, in consequence, be in the same condition as one in which the membrane formation has been induced by butyric acid but which has not been treated with the second corrective factor. It should show a membrane formation and a beginning of development, but should then perish.

The writer had observed in his earlier experiments on heterogeneous hybridization that when 80 or 100 per cent. of the eggs of *purpuratus* formed membranes upon fertilization with the sperm of starfish in hyperalkaline sea-water, often less than one per cent. of the eggs developed into larvæ, while the rest behaved as if only artificial membrane formation had been induced. Last winter the writer and Dr. Gelarie made sure that (as was already indicated by observations of Dr. Elder) only those eggs developed into larvæ in which a sperm nucleus was found, while the eggs which formed membranes without developing did not contain a sperm nucleus. The writer found, also, that when the concentration of NaHO and $CaCl_2$ used was comparatively high a smaller proportion of the eggs with membranes developed than when the concentration was low. This was easily understood on the assumption that the addition of NaHO as well as of $CaCl_2$ to the sea-water increased the adhesion of the starfish sperm to the jelly of the sea urchin egg, thus allowing the sperm to induce membrane formation, but preventing or rendering difficult its entrance into the egg.

It occurred to the writer that if this assumption was correct sea urchin eggs which had been deprived of the surrounding jelly by a treatment with hydrochloric acid should all develop when fertilized with starfish sperm and that they should no longer show a mere membrane formation without development. This was found to be true. Sea urchin eggs (*purpuratus*) were deprived of their jelly and several hours or a day later fertilized with starfish sperm in sea-water to which some $CaCl_2$ and NaHO had been added. Often as many as 50 per cent. of the eggs formed membranes and practically all developed into larvæ; while the eggs of the same female not deprived of jelly when fertilized under the same conditions would all form membranes, but with a very small percentage of eggs developing into larvæ. This indicates that Ca and NaHO may increase the adhesion of the spermatozoa of the starfish to the egg jelly of the sea urchin. It does not prove, however, that this increase of adhesive power is the factor by which the $CaCl_2$ and NaHO influence the entrance of the spermatozoon into the egg. It is possible that in addition these two substances also influence the surface condition of the egg by increasing the fluidity of the surface of the egg, thus favoring the spreading of the fertilization cone of the egg around the spermatozoa.

5. The question arises whether or not the addition of $CaCl_2$ and of bases favors the phenomenon of sperm agglutination[1] caused by the supernatant sea-water of the eggs of the same species, which F. Lillie has discovered. This is not very probable, since the addition of NaHO to sea-water shortens the duration of the agglutination[2] and therefore acts like an "antiagglutinin." It is true that the addition of $CaCl_2$ favors the agglutination, but so does the addition of $MgCl_2$; yet the latter substance without the presence of $CaCl_2$ or the addition of a base does not enable the spermatozoon to enter the egg.

It is, however, possible, if not probable, that some specific substance in the surface of the egg or spermatozoon or of both may also aid in the entrance of the spermatozoon into an egg of its own species. If this be true, in certain cases an excess of alkali or of $CaCl_2$ may compensate to some degree the lack of specific substances for the entrance of the spermatozoon into the egg, *e. g.,* in the fertilization of the egg of the sea urchin by the sperm of starfish, brittle stars, holothurians and others.

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[1] On the basis of observations on the sperm of *purpuratus* the writer was doubtful whether the specific cluster formation of the sperm caused by the supernatant sea-water of the eggs of the same species was a phenomenon of agglutination or a tropistic reaction. In *Arbacia* the agglutination is much more pronounced than in the case of *purpuratus*. The surface tension phenomena which the writer described may therefore find their explanation on the assumption of an agglutination, at least in the case of *Arbacia*.

[2] *The Journal of Experimental Zoology,* Vol. 17, page 123, 1914.

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PAROXYSMAL AURICULAR FIBRILLATION.

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The abnormalities of the cardiac mechanism which are responsible for various forms of cardiac arhythmia and disturbances in cardiac rate are now well understood, and have been especially well demonstrated by means of the string galvanometer. The analysis of disturbances of the cardiac mechanism, although of ever increasing diagnostic and therapeutic importance, is a field for original investigation which is fast losing its fertility. On the other hand, large problems lie before us in determining the essential causes of these cardiac disturbances. The progress of the treatment of cardiac disorders will be accelerated by the discovery of the now unknown changes, either within the heart or outside of it, which are responsible for these abnormalities of the cardiac mechanism. This is especially true of the large group of disorders characterized by disturbances in the generation of stimuli of the heart beat. These disturbances may predominate either in the auricles or in the ventricles, and may result in the occurrence of premature ectopic beats (extrasystoles), tachycardia of ectopic origin, the so-called auricular flutter or auricular fibrillation. These disturbances may occur in hearts already the site of anatomical lesions, but they may also occur in hearts which give no signs of such lesions.

It is with the hope that some light may be thrown on this question of the essential cause of disturbed excitability or stimulus formation that the following case of paroxysmal auricular fibrillation is reported.

CASE REPORT.

History.—I. G., Hospital No. 601, a jeweler, aged 50, born in Russia, was admitted to the hospital Feb. 23, 1912, complaining of cardiac palpitation and weakness. He knows of no cases of cardiac disease or rheumatism in his family, and the family history is otherwise negative. The patient had been a generally healthy man, but always "high strung" and rather nervous. He thought that he had neither scarlet fever nor diphtheria in childhood, but since the age of twelve he had frequent attacks of tonsilitis, and at the age of 15 typhoid fever. When 18 years old he had an apparently rather mild attack of gonorrhea, but there never had been any other venereal affection. His wife is living and well, and they have several healthy children. He had considerable distress after eating for many years, and had always taken large amounts of food, especially bread, and much tea. He used both alcohol and tobacco moderately and had never done laborious work. He never had rheumatism, but there was pain without swelling or redness in the right shoulder, beginning about eleven years ago and lasting fifteen months. He continued his occupation as a jeweler with very little interruption up to a few weeks before admission to the hospital.

The present illness began twelve years ago, in 1900, when the patient felt undue fatigue after exertion, and on the following day he had his first attack of cardiac palpitation and weakness, much like that for which he came to the hospital. He was in bed for three days at this time. He then had no such symptoms for about eighteen months, when the second attack occurred, and since then he has had similar attacks, usually lasting only a few hours, every four to six months. Occasionally they have lasted as long as three days. The patient thinks that the gastric disturbance, noticed especially after eating, had something to do with causing the attacks of palpitation. About eight weeks before admission the patient had the first of the series of attacks which brought him to the hospital. The first attack began Dec. 22, 1911, in the usual way, with a boring feeling in the epigastrium, a thumping feeling now and then in the heart, and finally a sudden onset of cardiac palpitation. There was also a feeling of pressure about the chest, and choking, headache and weakness. This attack lasted twenty hours and stopped suddenly. During the next three weeks the patient had two or three attacks of lesser severity, and then, Jan. 13, 1912, an attack began which lasted three and a half days. This was followed by four days when the patient felt quite well, at the end of which time the symptoms returned and continued for seven days. Then he was free from symptoms for ten days, when another attack, lasting a few days, occurred. Finally, after the patient had been feeling well for nearly two weeks, the final attack before admission began, and this time in an unusual way, a feeling of palpitation coming and going for a day or so, and then settling down on February 22, the day before admission, to what seemed to the patient a severe attack. Besides the symptoms that have been mentioned he had moderate pain in the left arm and in the back of the neck. He was able to walk into the hospital, but with some difficulty.

Physical Examination.—Physical examination showed the patient to be a rather poorly nourished, small man, who looked at least ten years younger than his age. He was lying quietly and apparently comfortably in bed, but appeared somewhat nervous. He was alert and highly intelligent. The eyes showed normal pupillary reactions to light and accommodation. The fundi were apparently normal except for slight tortuousness to the arteries. The tonsils were not enlarged. There was slight venous pulsation in the neck. The thyroid was not palpable. The lungs yielded normal physical signs throughout. There were no abnormal pulsations seen over the thorax, while there was a general heave in the region of the left nipple. The precordium was not prominent. The apex beat was in the fifth space, 10 cm. to the left of the midsternal line. The outline of cardiac dulness extended 4.0 cm. to the right of the midsternal line in the fourth space, and 11.5 cm. to the left of the fifth space. The heart sounds were clear throughout, no murmurs being heard. The second pulmonic was slightly accentuated, and had a liquid quality. The radial pulse was distinctly irregular, and had a rate of about 75 per minute. Many of the pulsations were well sustained, but they were irregular in both force and rhythm. The actual heart-rate as counted by auscultation over the heart was 126 per minute. The arteries were distinctly palpable in the arm, at the wrist and especially at the elbow. The temporal arteries were also indefinitely palpable. The abdomen was normal. Neither liver nor spleen was palpable. There was no edema of the extremities. Blood, hemoglobin 95 per cent. (Sahli) corrected reading = 119 per cent. Wassermann reaction was negative. The urine showed neither albumin nor casts. Its specific gravity was 1.030. The amount was 650 c.c. in twenty-four hours. Electrocardiograms showed that auricular fibrillation was present (Fig. 1).

Subsequent Course.—Digitalis was begun three days after admission, and in three days distinct slowing of the ventricles had occurred and the arhythmia was much less marked. March 1, at 10:30 a. m., the patient was bled from an arm vein and 40 c.c. of blood was obtained. At 11 a. m. he noticed a sudden change in the feeling of the heart beat, and declared that his attack was over. The examination revealed that the patient's heart was beating regularly, at first at a rate of 108, and in a few hours at about 90. The outline of relative cardiac dulness extended 4 cm. to the right in the fourth space, and 10 cm. to the left in the fifth space. The heart sounds were clear and of good quality all over the precordium. There were no murmurs, but the second aortic sound was accentuated. The patient felt immediately distinctly more comfortable, and seemed to be entirely well except for slight weakness. Electrocardiograms showed that the normal sequential beat had returned (Fig. 2).

During the night of March 6, after the heart had been beating normally for about sixty hours, the patient felt an occasional jumping feeling in the epigastrium, which he said usually occurred before attacks, and which led him on this occasion to predict the return of cardiac palpitation. During the night it was noticed that cardiac arhythmia existed for a few minutes on several occasions.

The heart continued to beat regularly, however, until March 10, when the patient awoke at 1 a. m. with the sensation of weakness and felt that his heart was beating irregularly. When examined at 9:30 a. m. the typical arhythmia of auricular fibrillation was present, the diagnosis of which was confirmed by electrocardiograms. This arhythmia continued until the afternoon of March 14, nearly five days. On this day the patient was restless, worried and especially uncomfortable in the morning, but in the afternoon the heart suddenly, practically instantly, became regular. The patient immediately felt much better, and except for muscular soreness and weakness was without symptoms. The cardiac examination gave results similar to those obtained during his first period of cardiac regularity. His condition remained excellent, and on March 22, after the heart had been regular for eight days, the patient was allowed to sit up out of bed for half an hour. March 23, a few minutes before 7 a. m., the pulse became suddenly irregular again. When seen a little later the patient was comfortable, and the heart, although completely irregular, was not beating very rapidly, and nearly all beats produced palpable radial pulsations. The heart sounds were clear. The arhythmia was still present on March 25, when the patient complained of feeling especially badly, with dizziness, weakness and a feeling of helplessness. On March 26, at about midnight, the patient awoke to find that his pulse was regular and rapid, and when examined the next morning the heart was beating perfectly regularly as before. The outline of relative cardiac dulness extended 2 cm. to the right and 11.5 cm. to the left in the fourth space, and the heart sounds were as before. The cardiac rate was 80. Electrocardiograms again showed that the auricles were beating normally, and were once more properly coordinated with the ventricles. The patient seemed in excellent condition without symptoms, and the heart maintained a rate of about 72 for several days. During the night of April 1, however, the patient had pain in the epigastrium and a feeling of suffocation, and on April 2, at 6 a. m., his pulse became suddenly irregular. When examined at 10 a. m., the heart was beating very irregularly, at a rate of about 140 per minute. Many beats failed to reach the wrist, and the radial pulse-rate was only about 100 per minute. The outline of relative cardiac dulness extended 3.5 cm. to the left in the third space, and 3.0 cm. in the fourth space, and 10.0 cm. to the left in the fourth space. The electrocardiograms were those of auricular fibrillation. From April 2 the cardiac arhythmia continued as long as the patient was under observation. The heart rate gradually slowed and became more nearly regular, but electrocardiograms showed that auricular fibrillation was still present when he was discharged on May 23, feeling quite comfortable and well. He had been walking about the ward for several days without symptoms. The arhythmia continued for several weeks after the patient left the hospital. It was subsequently learned that the patient died about two months later with symptoms suggesting acute dilatation.

DISCUSSION.

During the time the patient was in the hospital he had three periods of nine, nine and six days, when the heart was beating regularly, between which auricular fibrillation occurred for five and four days. It was also present during the first six days and during the last fifty-one days in the hospital.

The change from one form of cardiac mechanism to another was usually sudden, but premonitory symptoms were sometimes noted by the patient before the onset of the arhythmia. These were, as have been described, a feeling of gastric distress and a thumping feeling in the cardiac region which was probably caused by the occurrence of premature ventricular contractions (extrasystoles). The occurrence of such beats was recorded by electrocardiograms several times during periods of fibrillation. The actual onset of auricular fibrillation occurred each time after the patient had been asleep, once apparently during sleep. On the first occasion the patient awoke at 1:00 a. m. with a sensation of weakness and felt that his heart was irregular, and the second and third occasions the arhythmia began suddenly at 7:00 a. m. and 6:00 a. m., respectively. Muscular exertion and psychic excitement, unless it was in dreams, played, therefore, no rôle in initiating auricular fibrillation. Gastric disturbance also did not seem to be a factor at such times. Thus, there was no evidence supporting the patient's statement concerning the influence of gastric distress on the cardiac rhythm, which may be a factor in the production of complete arhythmia, as v.Müller[1] has pointed out.

Auricular fibrillation changed to the normal sequential beat three times while the patient was in the hospital. The first change occurred at 11:00 a. m., half an hour after 40 c.c. of blood had been withdrawn from an arm-vein, the second during the afternoon following an uncomfortable and restless forenoon, while the third change occurred at midnight, apparently during sleep. The changes were always sudden, and as the conditions under which they occurred were so different, no conclusions as to the possible cause of the changes can be drawn. The blood-letting was thought to have been a possible factor, but it was repeated on April 22, after fibrillation had been present three weeks, without any change in the cardiac mechanism. The patient stated that the return to the regular rhythm usually occurred when he was quiet.

The administration of digitalis may have been a factor in causing the disappearance of auricular fibrillation on two occasions. The drug was begun in the form of digipuratum tablets, 0.1 gm., three days after admission, five tablets the first day, decreasing one tablet each day. On February 29 the patient was nauseated after having had 1.5 gm. of digitalis, and the drug was discontinued at 3:30 p. m. The fibrillation ceased at 11:00 a. m. the next morning.

The drug was begun again in the same manner on March 11, two days after the onset of fibrillation, and continued for four days, when it was stopped after the sequential beat had returned. Small doses of the tincture, 0.2 c.c., three times a day, were given during this period of normal rhythm with the idea that it might assist in maintaining the regular beat, but on March 23 fibrillation again set in, and digipuratum was begun as before. This was continued for two days, and was discontinued after ten doses, as the patient complained of feeling dizzy and weak. Thirty-six hours after drug was discontinued, the normal beat again returned. The drug was withheld after the next onset of fibrillation had been present fifteen days, because it was felt that its administration probably had no effect in stopping the auricular fibrillation, and that the sequential beat would return spontaneously. As this did not occur, digipuratum was again administered. At this time and again later, it was given until nausea occurred, but produced no apparent effect on the auricular fibrillation. Digitalis was given primarily not with the idea of influencing the auricular activity, but because the rapid, irregular and insufficient ventricular activity present at the time of admission demanded it. In fact, as Mackenzie[2] and others have pointed out, digitalis may be apparently a factor determining the onset of auricular fibrillation. In our case fibrillation seemed prone to cease a day or two after the drug had been pushed to its physiological limit and discontinued. On one occasion fibrillation came on while small doses of the tincture were being taken.

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[1] Müller, v.: The Nervous Affections of the Heart, The Archives Int. Med., 1908, i, 1.

The ventricular activity was favorably influenced by digitalis, and the rapid, irregular heart beat was reduced to a rate of about 80, and became almost regular, while fibrillation continued. During the periods of regular cardiac activity the electrocardiograms showed that there was no prolongation of the time of conduction from auricles to ventricles, the P-R time in the second lead records averaging 0.17 second. This case does not lend support, therefore, to the suggestion put forward by Lewis[3] " that the slowing of the heart, when auricular fibrillation is present and digitalis is given, is due to an increase of a previously existing defect in the conduction to the ventricle of those impulses which are built up rapidly and irregularly in the auricle."

[2] Mackenzie: Digitalis, Heart, 1910–11, ii, 295.

[3] Lewis: Auricular Fibrillation and Its Relation to Clinical Irregularities of the Heart, Heart, 1909–10, i, 306.

BLOOD-PRESSURE.

The relation of changes in blood-pressure to changes in the cardiac mechanism was studied. The difficulty of determining the blood-pressure in cases of auricular fibrillation is evident, as the ventricular contractions, varying in force, produce variations in the blood-pressure from beat to beat. On account of the fact that exact estimations were impossible, the blood-pressure was not recorded at first, during auricular fibrillation, but later when the heart was beating more nearly regularly, the pressure was determined by the auscultatory method, at which the strongest beats produced sounds in the brachial artery and also at which practically all beats produced sounds. The average of these two readings was considered as the systolic blood-pressure. In a similar way two diastolic readings were taken and averaged. The blood-pressure readings are seen in the accompanying chart (Fig. 3). Although the blood-pressure was constantly high, marked changes took place, the systolic pressure varying from 125 to 215 mm. Hg. The diastolic pressure was more constant, but varied from 85 to 120 mm. Hg. In the second period of regular rhythm, from March 14 to 23, the blood-pressure rose gradually from 125 mm. Hg to 175, when fibrillation again set in. The blood-pressure did not fall with the onset of fibrillation, but twenty-four hours later it had fallen 25 mm. Hg, and remained relatively low until about thirty-six hours after the heart had become regular once more. Then the systolic pressure rose to 185 mm. Hg, remained high for three days, and then fell to 130 the day before fibrillation again set in. The blood-pressure was taken between 10:00 a. m. and 11:00 a. m. each day, and no observations were obtained immediately before and after a change in cardiac rhythm, so the day-to-day readings give no accurate indications as to changes in blood-pressure as a factor in causing changes in the cardiac mechanism. On the hypothesis that the high blood-pressure might be a factor in maintaining fibrillation, however, the patient inhaled 0.2 c.c. of amyl nitrite about three hours after the onset of fibrillation on March 23, at which time the systolic blood-pressure was 180 mm. Hg. He became nervous and dizzy, but except for the initial rapidity the heart beat did not change, and the blood-pressure was apparently not lowered. On another occasion (April

22), three weeks after the onset of fibrillation, the systolic pressure fell during the operation of blood-letting to 115 mm. Hg without any change in the cardiac mechanism. Erythrol tetranitrate and chloral were also administered during fibrillation, but without affecting appreciably the blood-pressure or the cardiac mechanism. On the other hand, the systolic pressure reached 215 mm. Hg on one occasion during fibrillation without any change in the beat. It may be said, therefore, that no relationship between changes in blood-pressure and changes in the cardiac mechanism can be made out. The blood-pressure varied greatly, both during auricular fibrillation and during the normal rhythm, but the blood-pressure was maintained generally at a high level, regardless of the form of cardiac activity present. These blood-pressure observations are in accord with those made by Lewis[4] on the experimental animal, in which he found that the onset of fibrillation was usually accompanied by a fall of blood-pressure, but the effect was inconstant. The circulation tended to accommodate itself to altered conditions, and to return to the same blood-pressure as that observed before the onset of fibrillation.

The effect of pressure on the neck over each vagus nerve was recorded by electrocardiograms several times, both during the normal rhythm and during fibrillation (Figs. 4–7). The abnormal susceptibility of the heart to mechanical stimulation of the vagi was striking. The length of time pressure wàs made over each nerve and the length of the responding pause may be tabulated as follows:

Auricular Fibrillation (Figs. 3 and 4).

	Pressure over right vagus. Seconds.	Ventricular stoppage. Seconds.	Pressure over left vagus. Seconds.	Ventricular stoppage. Seconds.
	3.0	3.3	3.4	4.8
	3.4	4.0	3.1	4.0
	2.8	4.0	3.6	4.4
	3.0	3.1	...	...
	3.0	3.4	...	...
Average	3.04	3.56	3.37	4.42
Proportion	1 :	1.17	1 :	1.22

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[4] Lewis: Fibrillation of the Auricles; Its Effects Upon the Circulation, Jour. Exper. Med., 1912, xvi, 395.

Normal Beat (Figs. 5 and 6).

	Pressure over right vagus. Seconds.	Ventricular stoppage. Seconds.	Pressure over left vagus. Seconds.	Ventricular stoppage. Seconds.
	1.4	3.2	2.0	3.6
	1.9	3.0	2.0	4.0
	1.9	3.0	2.0	1.9
Average	1.73	3.06	2.0	3.17
Proportion	1 :	1.77	1 :	1.53

Thus it is seen that neither during auricular fibrillation nor during the normal beat can any definite difference be made out between the effects of right and left vagus pressure, such as can sometimes be demonstrated in man (Robinson and Draper[5]), and as can be almost constantly shown with faradic stimulation of the nerves in the dog (Cohn,[6] Robinson[7]). The ventricles were stopped for a longer time proportional to the length of pressure when the heart was beating normally than when auricular fibrillation was present, whether the left or the right nerve was pressed on.

The mechanism responsible for the ventricular pauses which followed vagus pressure during auricular fibrillation is apparently different from that which brings about the stoppage of the normally beating heart. The electrocardiograms indicate that the activity of the fibrillating auricles was unaffected by pressure over the vagi, while they show that the normally beating auricles were brought to rest by this procedure. The manner by which vagus pressure brings about the long cessation in the ventricular activity in cases of auricular fibrillation has been discussed by Robinson and Draper,[5] and it was concluded that the ventricular pauses are apparently due to the blocking of stimuli from the auricles. There was no evidence as to whether this striking effect depends on an increased susceptibility of the conducting fibers to vagus stimulation or whether it depends on the stimuli from the fibrillating auricles being of such a nature that only slight lowering of the conductivity succeeds in blocking them.

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[5] Robinson and Draper: I. Studies with the Electrocardiograph on the Action of the Vagus Nerve on the Human Heart, Jour. Exper. Med., 1911, xiv, 217; II. Studies with the Electrocardiograph on the Action of the Vagus Nerve on the Human Heart, Jour. Exper. Med., 1912, xv, 14.

[6] Cohn: Jour. Exper. Med., 1912, xvi, 732.

[7] Robinson: The Influence of the Vagus Nerves on the Faradized Auricles in the Dog's Heart, Jour. Exper. Med., 1913, xvii, 429.

The possibility must also be considered of some quantitative change in the auricular activity which is not to be detected in the electrocardiograms, but which makes it incapable of stimulating ventricular contractions.

When the heart was beating normally there is practically no evidence that pressure over either vagus nerve lowered the conductivity, as the P-R time was never prolonged beyond 0.21 second after the cardiac stoppage in any of the records. The marked effect on the rate of the whole heart is in sharp contrast to the effect on conduction. It may be, however, that this effect on rate is responsible for the lack of effect on conduction, for during the cardiac standstill the conductivity of the heart is freed from the influence of functional fatigue, and so has its normal capabilities at the end of the cardiac pause. The harmonizing of the effects of vagus pressure on the heart during auricular fibrillation with that observed when the heart was beating normally is difficult.

The marked susceptibility of the heart to mechanical stimulation of the vagi may have been another expression of the cardiac abnormality which caused the auricular fibrillation. The hearts of patients with auricular fibrillation are often markedly affected by pressure over the vagi and an abnormal response to this procedure by a normally beating heart may possibly indicate that auricular fibrillation is more likely to occur in it than in a heart less affected by vagus pressure. It is difficult to say whether this abnormal response indicates a so-called heightened "vagus tone" or whether the heart itself is responsible for the marked response. A series of experiments were undertaken with the hope primarily that some new light might be thrown on the question of the relation of vagus activity and auricular fibrillation, but the results of the experiments[7] do not afford any positive evidence as to the rôle the vagi may play as a causative factor of auricular fibrillation in man. The dog's auricles may be thrown into fibrillation and tachycardia by direct faradization as readily after the vagi are cut as before, but vagus stimulation holds the auricles in their abnormal state of activity after it has been once set up by faradization.

The effect of atropin in our case was observed only during fibrillation and after 0.9 mg. atropin had been given hypodermically the ventricular rate increased markedly from 84 to 158 beats per minute, recorded by an electrocardiogram taken forty minutes after the administration of atropin. The waves of fibrillation were not so distinct in the records obtained during the rapid ventricular rate as before. The great increase in ventricular rate following atropin indicates that the vagi were constantly strongly active at this time in controlling the ventricular rate. A desire not to disturb the normally beating heart prevented the use of atropin during the periods of regular rhythm.

A series of electrocardiograms were made directly from various points on the chest wall during the normal beat and during fibrillation. The technic described by Lewis[8] was followed, in the hope of gathering further evidence regarding the significance of the waves of auricular fibrillation in the electrocardiograms. The results were unsatisfactory, as neither the P waves nor the waves of auricular fibrillation were distinct.

When this patient is considered from an anatomical rather than from a physiological viewpoint, it is found that the heart is normal as regards its size, position and the character of the heart sounds. No valvular murmurs were ever heard, and although the second aortic sound was somewhat accentuated, the muscle sounds were of good quality. The outline of cardiac dulness was measured repeatedly, and no changes in size as those observed by Lewis and Schleiter[9] could be made out, as an accompaniment of the changes in mechanism. A slight increase in the size of the heart between the time of admission and of discharge was noted, however, the total width increasing 1 cm. during this time.

The constantly high blood-pressure and the palpable arteries in the arms and temporal regions led to the diagnosis of arterial sclerosis. The urine gave no evidence of diseased kidneys, as the usual tests were repeatedly negative except for several hyaline casts on two occasions. The functional activity of the kidneys was tested

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8. Lewis: The Origin of the Electric Oscillations and the Direction of Contraction of the Ventricle in Instances of Complete Irregularity of the Heart (Auricular Fibrillation), Quart. Jour. Med., 1911, v, 11.

9. Lewis and Schleiter: The Relation of Regular Tachycardias of Auricular Origin to Auricular Fibrillation, Heart, 1912, iii, 173.

May 7 by the technic of Rowntree and Geraghty,[10] 6 mg. of phenolsulphonephthalein being injected intramuscularly. The first appearance of the drug in the urine was not definitely determined, but was estimated at about ten minutes. During the first hour 36.8 per cent. and during the second hour 21.9 per cent., or a total for two hours of 58.7 per cent. of the drug was excreted. Thus the functional activity of the kidneys was but slightly below the normal limits of excretion (40–60 per cent. the first hour, and 60–85 per cent. in two hours). The sole definite anatomical lesion, therefore, is apparently arterial sclerosis.

Arterial sclerosis is frequently a prominent characteristic of cases of auricular fibrillation. Thus Lea[11] found it present in 57.1 per cent. of the non-rheumatic cases in his series. No definite change in the cardiac muscle has been described in these cases with sufficient frequency to allow any conclusions to be drawn as to an anatomical basis for auricular fibrillation, and the post mortem examinations of some of the hearts in which fibrillation had been present reveal hearts which are anatomically sound (Gossage and Hicks[12]). Lewis[8] believes that it is changes in the walls of the auricles themselves which probably cause fibrillation, and he has expressed the opinion that anemia of the auricular walls may be a factor in setting up fibrillation. Changes in the blood-supply of the auricular walls may well have been an important factor in causing the attacks of fibrillation in our patient. The evident arterial sclerosis and the high but markedly changing blood-pressure might readily have caused abnormalities in the cardiac blood-supply, which not only rendered the auricles more prone to fibrillation and abnormally susceptible to vagus stimulation, but also furnished the immediate cause of an attack of auricular fibrillation by a temporary diminution of the cardiac circulation. There is very little in support of this hypothesis, but arterial sclerosis and high blood-pressure have been prominent in several cases of transitory auricular fibrillation which have been observed. Thus Hornung[13] reports three cases in which these abnormalities were prominent features, and arterial sclerosis was also mentioned in two of Fox's[14] six cases. The case reported by Lewis and Schleiter, although only 28 years old, had somewhat thickened arteries, but on the one occasion recorded the blood-pressure was low.

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[10] Rowntree and Geraghty: An Experimental and Clinical Study of the Functional Activity of the Kidneys by Means of Phenolsulphonephthalein, Jour. Pharm. and Exper. Ther., 1909–10, i, 579.

[11] Lea: Some Points in Relation to the Etiology of Auricular Fibrillation, Quart. Jour. Med., 1911, iv, 423.

[12] Gossage and Hicks: On Auricular Fibrillation, Quart. Jour. Med., 1913, vi, 435.

The paroxysms of auricular fibrillation may occur over a period of many years, and our patient had them apparently for twelve years. One of Hornung's cases suffered with attacks of cardiac arhythmia for five years and another for at least ten, while one of the cases reported by Fox, the case originally observed by Cushing and Edmunds, had attacks for over twenty years. These cases, therefore, do not always deserve the bad prognosis which is usually accorded to auricular fibrillation. They may have anatomically normal hearts and be closely allied to cases of paroxysmal tachycardia, as Lewis and Schleiter point out. The prognosis may not necessarily be more grave than in tachycardia. However, the auricular fibrillation tends to become permanent, as Fox's cases well illustrate, and this is what happened in our case, as fibrillation was present during the last two months that he was in the hospital, and continued until his death. about two months after his discharge. There is, therefore, apparently a group of cases which may be termed paroxysmal auricular fibrillation, in which arterial sclerosis may be an underlying cause of the abnormal cardiac activity, and in which attacks of fibrillation may recur over a period of many years in patients with anatomically normal hearts, without great impairment to the general health.

SUMMARY.

A case is reported in which transient attacks of auricular fibrillation were observed and recorded by electrocardiograms. These attacks had apparently occurred over a period of twelve years, and auricular fibrillation became permanent while under observation. No definite cause could be discovered as initiating the attacks. No definite relation could be established between changes in blood-pressure, which was always abnormally high and variable, and the changes in the cardiac mechanism. The heart was strikingly susceptible to pressure over each vagus nerve, both during fibrillation and during the normal cardiac activity. This abnormality may be another expression of the changes causing auricular fibrillation. Electrocardiographic records of these vagus effects were obtained.

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[13] Hornung: Über atypische Tachykardische Paroxysmen, Deutsch. Arch. f. klin. Med., 1907, xci, 469.

[14] Fox: The Clinical Significance of Transitory Delirium Cordis, Am. Jour. Med. Sc., 1910, cxl, 815.

The heart of the patient gave no signs of an anatomical lesion, but arterial sclerosis, a frequent accompaniment of auricular fibrillation, was present. The presence of this lesion and the high, inconstant blood-pressure suggest the possibility that changes in the cardiac blood-supply may have been the underlying causative factor in this case. Other cases showing attacks of auricular fibrillation occurring over a period of years have been reported in which high blood-pressure and arterial sclerosis were prominent features. These cases seem closely allied to paroxysmal tachycardia, and although the auricular fibrillation tends to become permanent, the prognosis for them is not necessarily so grave as that which is given in ordinary cases of auricular fibrillation with outspoken cardiac lesions.

EXPLANATION OF PLATES.

Fig. 1.—Curves 192. February 25. First, second and third leads, showing auricular fibrillation. Ventricular rate averages 112 beats per minute. The time marking is always in 1/5 seconds.

Fig. 2.—Curves 205. March 4. First, second and third leads obtained during the first period of cardiac regularity. The records show that the normal sequential beat is present. P.-R. time, second lead, average 0.17 second. Rate averages seventy-nine beats per minute.

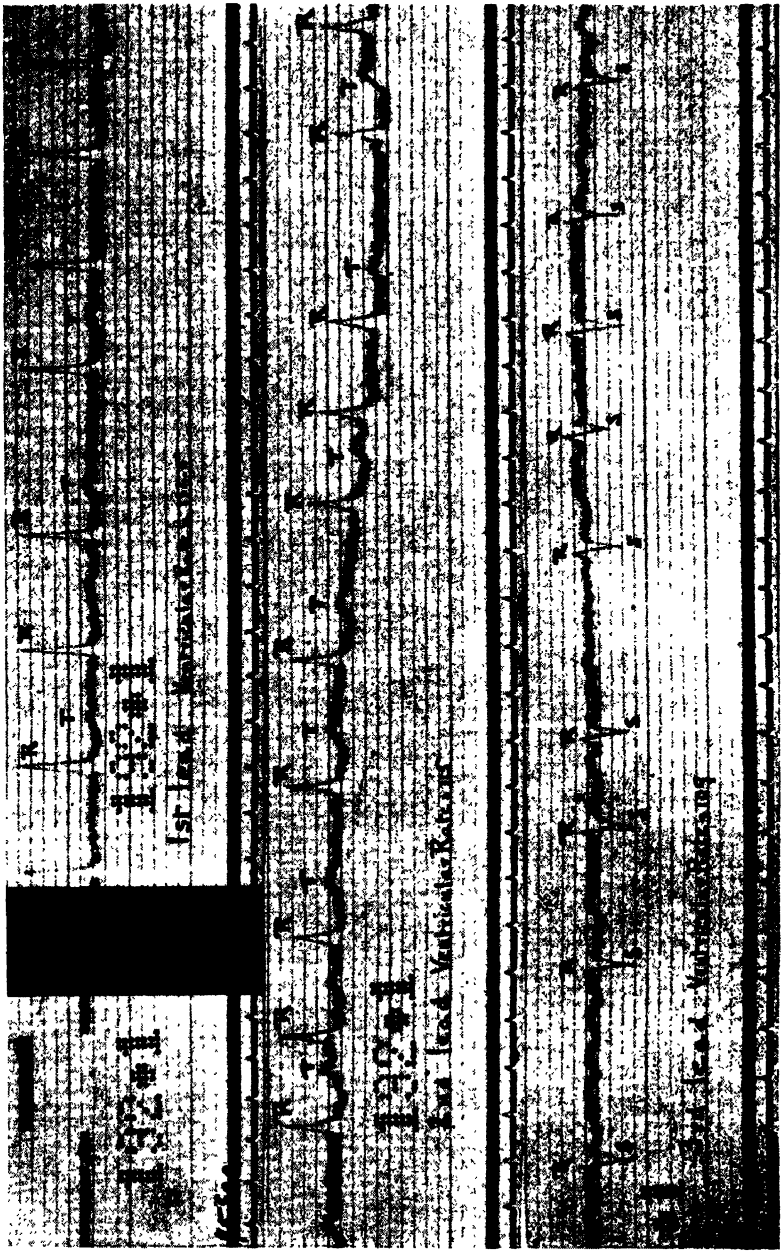
Fig. 3.—The systolic (upper line) and the diastolic (lower line) blood-pressure. The type of cardiac activity and the drug administration are also indicated.

Fig. 4.—Curve 198.7. February 28. The effect of pressure for three seconds over the right vagus during auricular fibrillation.

Fig. 5.—Curve 198.8. February 28. The effect of pressure for 3.4 seconds over the left vagus during auricular fibrillation.

Fig. 6.—Curve 205.3. March 4. The effect of pressure for 1.4 seconds over the right vagus on the normally beating heart.

Fig. 7.—Curve 205.5. March 4. The effect of pressure for 2 seconds over the left vagus on the normally beating heart.



[redacted]

FIG

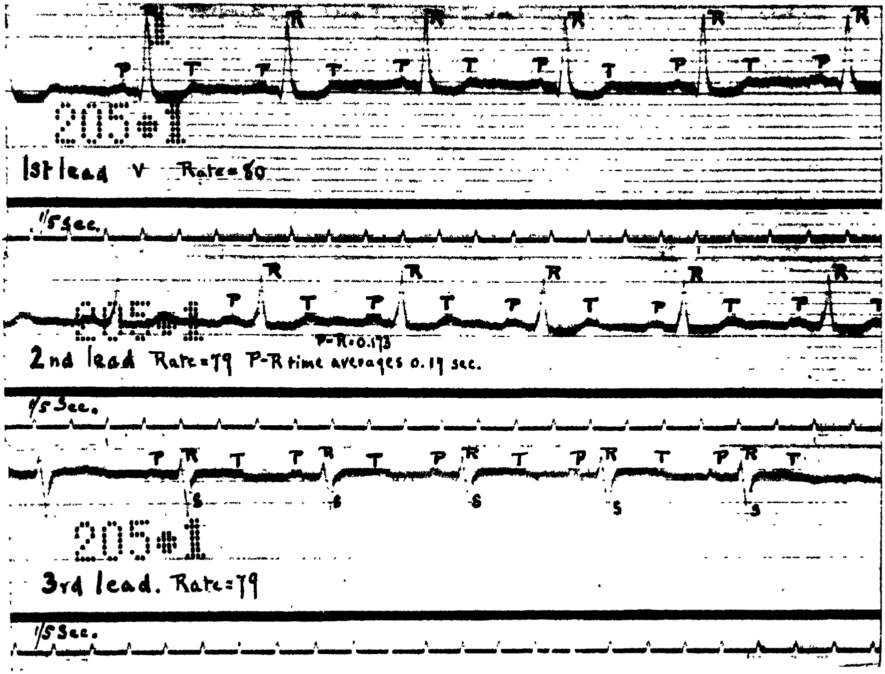

P
R
T
205#1
1st lead V Rate=80
1/5 Sec.
P
R
T
205#1
P-R=0.173
2nd lead Rate=79 P-R time averages 0.17 sec.
1/5 Sec.
P
R
T
S
205#1
3rd lead. Rate=79
1/5 Sec.

FIG. 2.

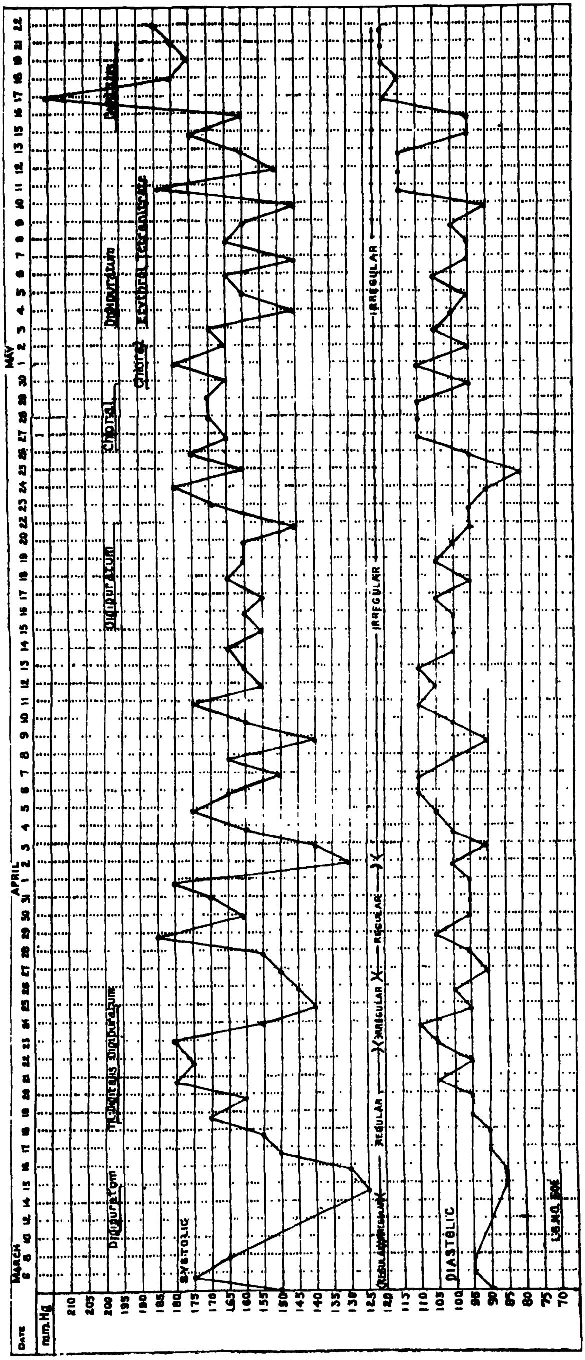

Date
mm.Hg
MARCH
APRIL
MAY
210
205
200
195
190
185
180
175
170
165
160
155
150
145
140
135
130
125
120
115
110
105
100
95
90
85
80
75
70
SYSTOLIC
DIASTOLIC
Digipuratum
Tr. Digitalis Digipuratum
Digipuratum
Chloral
Chloral
Digipuratum
Erythrol tetranitrate
REGULAR
IRREGULAR
REGULAR
IRREGULAR
REGULAR
IRREGULAR
IRREGULAR
L.B.NO. 596

FIG. 3.

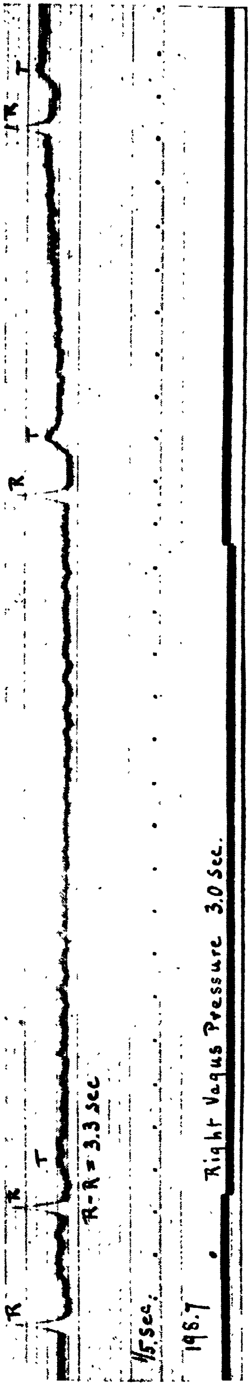

R
T
R
T
R
T
R-R = 3.3 sec
1/5 sec.
196.7
Right Vagus Pressure 3.0 Sec.

FIG. 4.

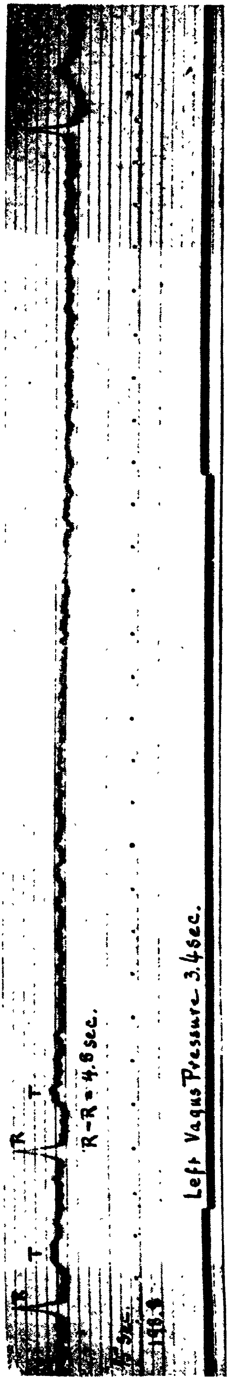

R
T
R
T
R-R = 4.8 sec.
1/5 sec.
Left Vagus Pressure 3.4 sec.

FIG. 5.

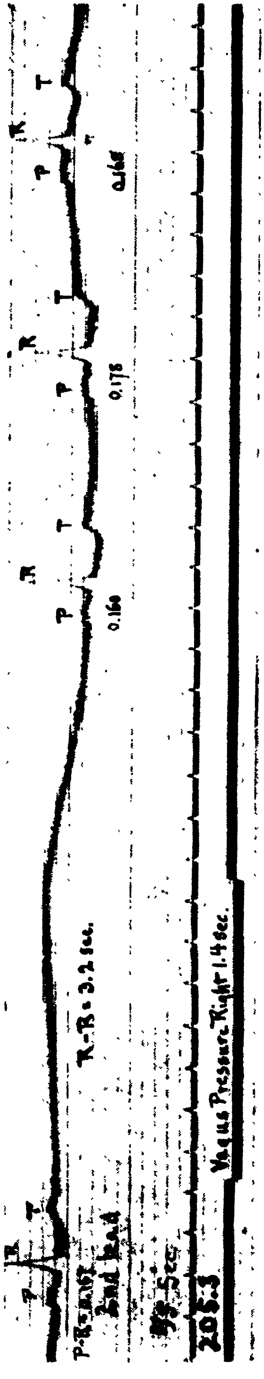

P-R = 0.157
2nd lead
1/5 Sec.
205.3
P
R
T
R-R = 3.2 sec.
Vagus Pressure Right 1.4 sec.
0.160
0.178
0.168

FIG. 6.

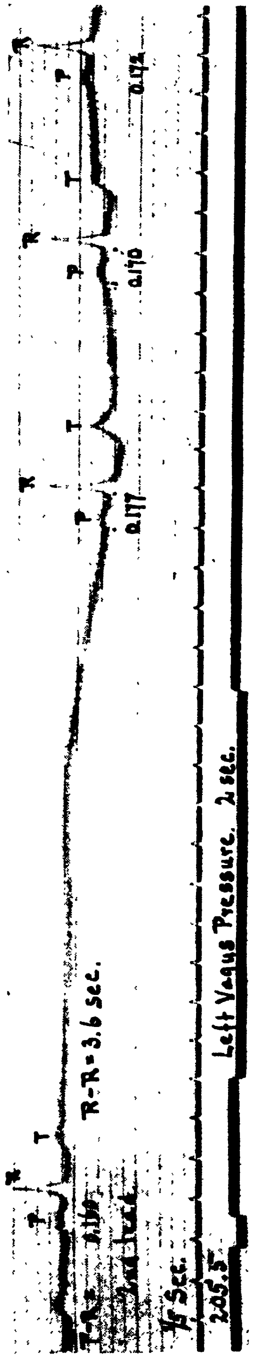

P-R = 0.160
2nd lead
1/5 Sec.
205.5
P
R
T
R-R = 3.6 sec.
Left Vagus Pressure. 2 sec.
0.177
0.170
0.172

FIG. 7.

[Reprinted from HEART, February, 1914, Vol. v, No. 2, pp. 141–146.]

THE OCCURRENCE OF AURICULAR CONTRACTIONS IN A CASE OF INCOMPLETE AND COMPLETE HEART-BLOCK DUE TO STIMULI RECEIVED FROM THE CONTRACTING VENTRICLES.

BY ALFRED E. COHN AND FRANCIS R. FRASER.

(From the Hospital of The Rockefeller Institute for Medical Research.)

In a patient sixty-two years of age, probably suffering from syphilis, we were able to demonstrate the presence of incomplete auriculo-ventricular heart-block (Fig. 1). During a long period of observation we noticed a frequent change from incomplete block to complete auriculo-ventricular dissociation. We were able to satisfy ourselves that the change in the heart's rhythm depended in great part on the fluctuation in the rate of the auricles in the same way as has been reported by Erlanger[5] in a patient and by Erlanger and Hirschfelder[6] in experiments. During the periods when the rhythm was one of complete dissociation, we noticed that the ventricles were often followed by small waves easily distinguished in the electrocardiograms. These we have identified as the representatives of auricular contractions resulting from stimuli received from the contracting ventricles. It is the occurrence of these waves and certain facts related to them that we wish to report.

Apart from auricular contractions, which occurred normally in this patient, an abnormal form also appeared. The abnormal complexes were found only after independent ventricular contractions, never after sequential cycles, the interval between ventricular and auricular representatives being 0.1 to 0.14 seconds. The conduction time of stimuli passing in the normal direction was most often 0.2 seconds, though sometimes it was slightly less. In the electrocardiograms (Fig. 2, 3, 5 and 6), these waves are represented by small acute (1 to 1.5 mm.) deflections directed downwards. They are obviously auricular contractions and result from ventricular beats which stimulate the auricles to contraction. A single wave of the kind described is seen in Fig. 2. The time at which it appears is just before a normal (sinus) auricular contraction is expected. It is the only point in the curve where the regularly recurring upright auricular complex fails to appear. In its place is an auricular beat represented by a negative wave of the kind described. In all the curves which were taken from this patient, these inverted waves appeared only shortly before the normal (upright) ones were expected; that is to say, they appeared only in the late, but never in the early portion of the diastoles of normal auricular contractions. If both a normal and a sequential auricular contraction became due during the period of late diastole, then the normal beat invariably occurred and the abnormal one was suppressed (* in Fig. 3).

A comparison of Fig. 4 and 5 illustrates these points. In Fig. 4 no auricular beats follow the contractions of the ventricles. Of the three ventricular complexes shown, the first and third are preceded by normal auricular beats, but none is seen in relation with the second. An auricular contraction is due and must indeed have occurred at the same time as the middle ventricular beat, for this *R* wave is 2 to 3 mm. taller than the other two. The normal auricular beats occurred then in close proximity to the three ventricular systoles, and stimuli to which the contractions of the ventricles gave rise reached the auricles when they were refractory. In Fig. 5 the interval between the normal (upright) auricular representations is equal to two of those seen in Fig. 4. If the remaining ones were present, they would fall a little more than 0.2 seconds after the beginning of the ventricular complexes; that is to say, auricular diastole would terminate 0.2 seconds after the beginning of ventricular systoles. But before this length of time has elapsed, stimuli from the ventricles have reached the auricles and have occasioned contractions which are represented by negative *P* waves. Such waves appear after each of the ventricular complexes in Fig. 5. They have forestalled the normal auricular beats and have taken their places.

Auricular contractions of similar form and similarly situated were observed experimentally by one of us.* In dogs morphine was injected intravenously, on the plan of v. Egmond[3] and of Einthoven and Wieringa,[4] to stimulate the cardio-inhibitory mechanism. Under these circumstances the rate of the auricles is reduced so that it is sometimes slower than that usually developed inherently by the ventricles. Instances in which the auricular rate was only 11 per minute were observed. The independent ventricular contractions which arose often occasioned stimuli which caused auricular contractions like those seen in this patient. An instance where the auricular beats of the variety under discussion appeared, when the rate of the normal auricular contractions was very slow, is seen in Fig. 6, taken from our patient, where the reduction in the auricular rate was due to vagus pressure.

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* A report dealing with this subject appears in The Journal of Experimental Medicine, 1913, XVIII, 715.

DISCUSSION.

In connection with the occurrence of auricular contractions as the result of stimuli arising in the ventricles, several matters of interest deserve consideration. The first relates to the form of the electrocardiographic waves. In our patient and also in the experimental curves to which we have referred, the auricular curves are downwardly directed deflections. Lewis[8] showed in his experiments that waves of this outline usually resulted when the stimulus to contraction spread from a focus situated at the lower levels of the auricular muscle. More recently, Ganter and Zahn[7] have attempted to show in a preliminary publication that, if the auricular contraction is represented by an inverted wave, the stimulus to contraction arises in the auricular portion of the auriculo-ventricular node—a portion identical with that described by one of us[1] as the auriculo-nodal junction. In general, experimenters are agreed that when contractions spread from the lower part of the auricular musculature, the wave in the electrocardiogram is likely to be inverted. In our own case the contractions seem to have spread in the same way, but did so in response to stimuli which arose in the walls of the ventricles.

We have stated that unless the ventricular stimuli occurred late in the normal auricular diastoles, no abnormal auricular contractions resulted. In Fig. 3 it is quite clear that an abnormal beat failed to appear at a point 0.37 seconds (P-$R = 0.24$ plus R-P in this curve

= 0.13 seconds) after the preceding normal auricular contraction. In the same curve, a negative *P* wave did occur 0.6 seconds after a normal *P*, so the refractory period of the auricle is certainly less than this length of time (0.6 seconds). Although we know that the refractory period is at least 0.37 seconds, our curves do not permit us to state exactly how much longer it is. Its length lies between 0.37 and 0.6 seconds. Another point suggested by Fig. 2 must be considered. The last interauricular interval reproduced measures 0.75 seconds and it ends 0.27 seconds after the beginning of the preceding *R* wave. According to the other examples, a negative *P* wave was expected 0.13 seconds before this time had elapsed. A satisfactory explanation for its absence cannot be given. But we have already shown an instance (*in Fig. 3) where a normal beat and an abnormal one are due simultaneously, and the normal one prevails. Here, as elsewhere in these curves, the normal beat not only prevails when both are expected together, but frequently also when the abnormal beat falls in what may be called its presystolic period. But that this is not always the case is seen in Fig. 5, where the retrograde beats appear, though normal ones are due directly afterwards. It is possible that the question of the auricular rate, as Trendelenburg[10] has shown, so different in the two examples reproduced, may have an influence upon the occurrence of one or other type.

The failure of the auricles to respond to the stimuli from the ventricles early in diastole may be attributed to one of two causes. The irritability of the auricular muscle may not have recovered to a sufficient degree for it to respond to a stimulus of the strength supplied by the contraction of the ventricles. Or, stated in another way, the auricles remain refractory to normal stimuli for longer periods than are observed in experimental conditions. The refractory period has, in fact, been shown by Trendelenburg[9] to be somewhat longer in the auricles than in the ventricles, but these results were obtained from experiments upon frogs' hearts, and the results apply to electrical stimuli. Experiments more nearly approximating the normal conditions in mammal or in human hearts are unknown to us. We must conclude that contractions resulting from physiological stimuli can occur only in a much later portion of auricular diastole than is the case when artificial excitations are employed.

Although the abnormal auricular beats which we have described follow invariably on ventricular beats which immediately precede them, we have not ventured to describe them as being retrograde in the sense that the stimuli arising in the ventricular walls passed in the reverse direction over the auriculo-ventricular bundle. It is their occurrence in a case of heart-block which has made us hesitate to give them this designation, even though we know that the bundle was capable of conducting impulses in this patient. For records from another patient* have since come to our notice, in which similar waves appeared, and this patient during four or more years of constant observation has invariably been the subject of complete heart-block. A consideration of the curves from this patient induces us to consider the possibility that the abnormal auricular beats in both patients were due to the mechanical stimulus of the contracting ventricular mass acting upon the auricular tissues.

SUMMARY.

We have described, in a case of heart-block which was sometimes complete and sometimes incomplete, the occurrence of auricular contractions due to stimuli received from the contracting ventricles, while auricles and ventricles were beating in complete dissociation. The case belongs to a group in which the rhythm of the heart depended in large measure on the rate of the auricles.

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3 v. EGMOND (A. A. J.). "Ueber die Wirkung des Morphins auf das Herz (zugleich ein Beitrag zur Frage der Morphingewohnung)." Archiv f. exper. Pathol. u. Pharmakol., 1911, LXV, 199–213.

4 EINTHOVEN (W.), and WIERINGA (J. H.). "Ungleichartige Vaguswirkung auf das Herz, elektrokardiographisch untersucht." Archiv f. d. ges. Physiol., 1913, CXLIX, 48–64.

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* These records were shown to one of us (A. E. C.) by Dr. John Parkinson and were taken by him from one of Dr. James Mackenzie's patients at the London Hospital. Dr. Parkinson will describe this case later.

[5] Erlanger (J.). "On the physiology of heart-block in mammals, with especial reference to the causation of Stokes-Adams disease." Journ. Exper. Med., 1905, vii, 676.

[6] Erlanger (J.), and Hirschfelder (A. D.). "Further studies on the physiology of heart-block in mammals." Amer. Journ. Physiol., 1905–6, xv, 153–206.

[7] Ganter and Zahn. "Ueber das Elektrokardiogramm des Vorhofs bei monotoper und heterotoper Automatie." Verhandl. d. deutsch. Kongr. f. inn. Med., 1913, xxx, 274–278.

[8] Lewis (Th.). "Galvanometric curves yielded by cardiac beats generated in various areas of the auricular musculature. The pacemaker of the heart." *Heart*, 1910–11, ii, 23–41.

[9] Trendelenburg (W.) and Fujita (T.). "Ueber den zeitlichen Ablauf der Refraktärphase am Herzen." Archiv f. d. ges. Physiol., 1911, cxli, 378–388.

[10] Trendelenburg (W.). "Untersuchungen über das Verhalten des Herzmuskels bei rhythmischer electrischer Reizung." Archiv f. Anat. u. Physiol., Phys. Abth., 1903, 271–310.

Fig. 1. *I, II* and *III*. Incomplete heart-block (2:1).

Fig. 2. Lead *II*. A single retrograde (ventricular) auricular systole and a single co-ordinated cycle is seen in a stretch of curve otherwise showing complete dissociation.

Fig. 3. Lead *II*. Taken during an atropine test. The figure shows complete dissociation. One auricular systole is due to a retrograde (ventricular) stimulus.

Fig. 4. Lead *II*. Taken during digitalis intoxication. Complete dissociation is shown.

Fig. 5. Lead *II*. Taken during digitalis intoxication. The figure shows complete dissociation. Auricular contractions due to retrograde (ventricular) stimuli are seen.

Fig. 6. Lead *III*. The arrows indicate the onset and offset of pressure over the right vagus nerve.

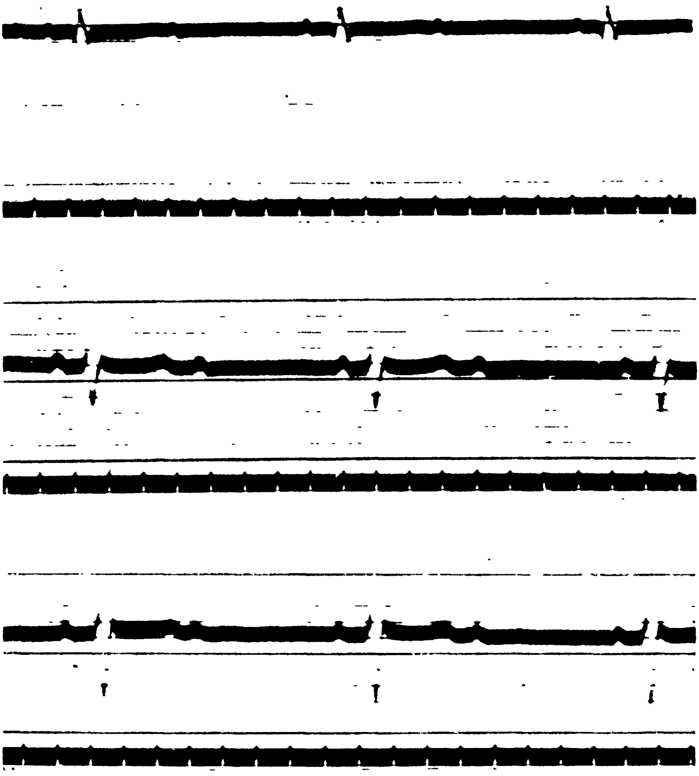

Fig. 1

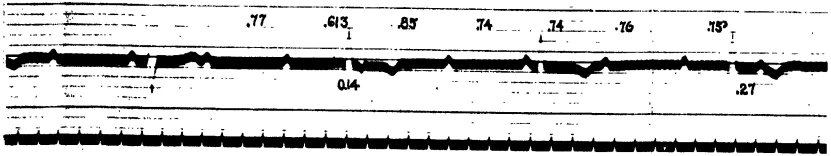

Fig. 2.

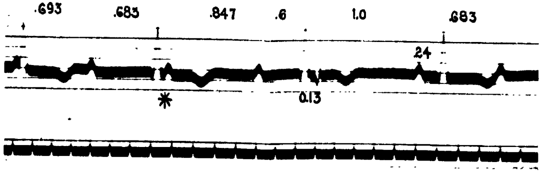

Fig. 3

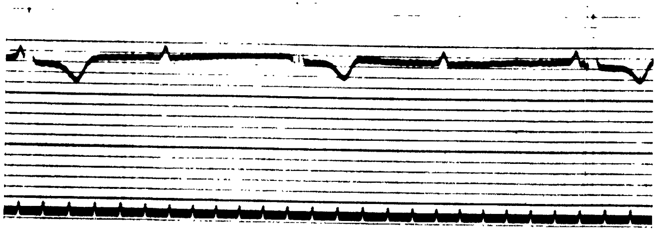

Fig. 4.

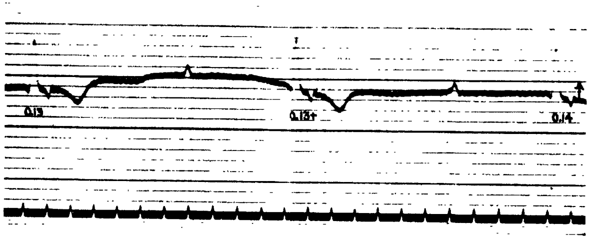

Fig. 5.

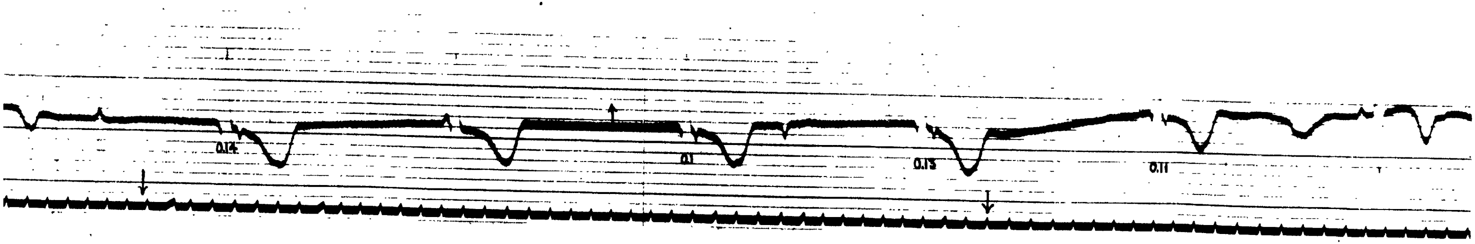

Fig. 6.

[Reprinted from THE JOURNAL OF EXPERIMENTAL MEDICINE, March 1, 1914, Vol. xix, No. 3, pp. 303–308.]

THE INDOPHENOLOXYDASE CONTENT OF TISSUES FROM RABBITS INFECTED WITH PNEUMOCOCCUS.*

BY FLORENTIN MEDIGRECEANU, M.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

The property of animal tissues to oxidize a mixture of naphthol and paraphenylendiamine into indophenol was first demonstrated by Römann and Spitzer.[1] Since then it has frequently been used as a test for oxydases, both in plants and animals. Recently Vernon[2] has devised a method for using this reaction quantitatively and has applied it to a number of problems, such as the quantitative distribution of the indophenoloxydase in various animal tissues, the relation of its action to lipoids, etc. According to Battelli and Stern,[3] indophenoloxydase belongs to the group of oxidizing agents which probably play an important part in the respiratory processes in the tissues. They call these oxidizing agents oxydones. The oxydones are not washed away from the tissues by water; alcohol and trypsin destroy them easily; the maximum activity occurs at 40° C.

It seemed of interest to study the behavior of the indophenoloxydase of animal tissues during pneumococcus infections. The problem has a bearing on the changes in the oxidation processes occurring in infectious diseases generally. In the present paper are recorded comparative figures showing the indophenoloxydase content of tissues from rabbits that died of general pneumococcus infection and of tissues from normal control animals.

METHOD.

The rabbits were given intravenously 0.2 to 0.5 of a cubic centi-

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* Received for publication, January 15, 1914.

[1] Römann, F., and Spitzer, W., *Ber. d. deutsch. chem. Gesellsch.*, 1895, xxviii, 567.

[2] Vernon, H. M., *Jour. Physiol.*, 1911, xlii, 402; 1911–12, xliii, 96; 1912–13, xlv, 197; *Biochem. Ztschr.*, 1912, xlvii, 374.

[3] Battelli, F., and Stern, L., *Compt. rend. Soc. de biol.*, 1913, lxxiv, 212.

meter of a freshly prepared virulent pneumococcus broth culture. Death followed usually after sixteen to twenty-two hours. The normal control animals were killed by a blow on the neck at the same time that the infected rabbits died, or within one to two hours later. The organs from both the infected and the normal animals were taken out, prepared, and kept under the same conditions.

The estimation of the indophenoloxydase content was made according to Vernon's[4] method. Five cubic centimeters of the reagent mixture, containing in 100 cubic centimeters a solution of M/1,500 α naphthol and paraphenylendiamine and M/620 sodium carbonate, are exposed to the action of 0.5 of a gram of the finely chopped tissue in Petri dishes, 8.8 centimeters in diameter, with frequently repeated stirring. The reagent solutions are mixed immediately before their addition to the minced tissue, and for each test a fresh pipette is used.

The time of exposure was one half hour for the renal cortex and the heart muscle, and one hour for the liver tissue. It was found that this period of time is sufficient to obtain definite information in regard to the activity of the indophenoloxydase present.

The indophenol formed is then extracted with ten cubic centimeters of 96 per cent. alcohol for one half hour, filtered, and its quantity calorimetrically estimated by diluting a certain number of cubic centimeters of it with 50 per cent. alcohol to the depth of color of the standard solution. Test-tubes of the same diameter and thickness must be used. The standard solution is prepared by diluting 1.4 cubic centimeters of the above mentioned reagent mixture to 200 cubic centimeters with 50 per cent. alcohol and keeping it three or four days, or until the maximum of color is reached. It contains 0.01 of a gram of indophenol in 100 cubic centimeters. The standard solution is renewed every week.

A large number of experiments have been made under various conditions. The more constant and reliable results have been obtained with liver, renal cortex, and heart muscle. They are recorded in the following tables, where other details of the experiments are also given.

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[4] Vernon, H. M., *loc. cit.*

TABLE I.[5]

Pneumococcus Rabbit Died 22 Hours after the Intravenous Injection. Pneumococci Present in Blood. Normal Control Rabbit Was Killed at the Same Time. Percentage of Indophenol Formed.

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
Tissues kept in ice chest						
13.4[6]	6.0	11.5	7.9	17.5	15.7	10 to 11 hrs. after death.
9.1	<3.0	10.6	7.2	17.5	12.1	30 to 31 hrs. after death.
12.1	<3.0	12.7	6.0	—	—	3 days later.
Tissues autolyzed at 37° C.[7]						
12.1	4.0	10.3	7.2	17.0	15.7	10 to 11 hrs. after death. Autolysis at 37° C. lasted for 1 hr.
9.1	<3.0	8.4	<3.0	12.7	12.1	30 to 31 hrs. after death. Autolysis at 37° C. of the liver and kidney tissues lasted for 2½ hrs., of the heart muscle for 5 hrs.

TABLE II.

Pneumococcus Rabbit Died 22 Hours after Injection. Pneumococci Present in Blood. Normal Control Rabbit Was Killed 1 Hour Later. Percentage of Indophenol Formed.

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
Tissues kept in ice chest						
13.4	6.0	11.5	7.9	17.5	15.7	10 hrs. after death.
9.1	<3.0	10.6	7.2	17.5	15.7	30 hrs. after death.
6.6	<3.0	11.5	5.4	—	—	15 dys. later.
Tissues autolyzed at 37° C.						
12.1	4.0	10.3	7.2	17.0	15.7	10 hrs. after death. Autolysis at 37° C. lasted for 1 hr.
9.1	<3.0	8.4	<3.0	12.7	12.1	30 hrs. after death. Autolysis at 37° C. of the liver and kidney tissues lasted for 2½ hrs., of the heart muscle for 5 hrs.
6.0	<3.0	9.0	<3.0	—	—	15 dys. later. The tissues have been kept in the ice chest since the last experiment.

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[5] The temperature at which the figures recorded in these tables were obtained varied between 22° and 28° C. Each normal control experiment was made at the same time and under the same conditions as the pathological.

[6] The figures that follow are not corrected for the spontaneous oxidation of the reagents into indophenol. On account of the short duration of the experiments (one half to one hour) the quantity formed is negligible.

[7] The autolysis at 37° C. was carried out in small Petri dishes 4 cm. in diameter, hermetically sealed with vaselin, in order to prevent the evaporation of the tissue water.

TABLE III.

Two Rabbits Have Been Inoculated Intravenously with Pneumococcus Broth Culture. Death Followed after 40 and 41 Hours Respectively. Normal Control Rabbit Was Killed 1 Hour Later.

Percentage of Indophenol Formed.

Renal cortex.			Heart muscle.		Remarks.
Normal rabbit.	Pneumococcus rabbit.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
15.7	15.0	15.0	15.7	12.0	22 and 24 hrs. after death. Tissues kept in ice chest.
9.1	<3.0	<3.0	4.2	<3.0	22 and 24 hrs. after death. Autolyzed at 37° C., kidney tissue for 7 hrs., heart muscle for 19 hrs.

TABLE IV.

Pneumococcus Rabbit Died 16 Hours after Injection. Pneumococci Present in Blood. Normal Control Rabbit Was Killed 2 Hours Later.

Percentage of Indophenol Formed.

Liver.			Renal cortex.			Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture.[8]	Pneumococcus rabbit.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	Pneumococcus rabbit.	Normal rabbit.	Pneumococcus rabbit.	
9.1	—	10.9	10.9	—	9.6	13.9	12.0	10 to 12 hrs. after death. Tissues kept in ice chest.
<3.0	<3.0	<3.0	9.1	9.1	4.2	10.2	7.2	10 to 12 hrs. after death. Tissues autolyzed at 37° C., liver and kidney for 3 hrs., heart muscle for 9 hrs.

TABLE V.

Normal Rabbit. All Tissues Were Kept at Room Temperature, 23° to 28° C.

Percentage of Indophenol Formed.

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	
15.0	15.0	10.8	10.6	18.6	18.6	6 hrs. after death.
10.6	10.6	7.0	7.1	13.8	17.4	20 hrs. after death.
9.6	8.0	7.0	6.0	—	—	44 hrs. after death.
<3.0	<3.0	<3.0	<3.0	9.0	9.0	6 dys. after death.

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[8] One drop of a freshly prepared pneumococcus broth culture was added to the normal tissue in all similar experiments.

TABLE VI.

Normal Rabbit.

Percentage of Indophenol Formed.

Renal cortex		Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	
14.1	—	27.0	—	6 hrs. after death. Kept in ice chest.
10.5	12.0	14.0	13.5	6 hrs. after death. Autolyzed at 37° C. for 3 hrs.

TABLE VII.

Normal Rabbit.

Percentage of Indophenol Formed.

Liver.		Renal cortex.		Heart muscle.		Remarks.
Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	Normal rabbit.	Normal rabbit plus pneumococcus culture.	
Tissues kept in ice chest						
17.7	—	10.3	—	—	—	8 hrs. after death.
18.0	—	10.3	—	22.7	—	14 hrs. later.
19.0	—	10.5	—	19.0	—	5 dys. later.
Tissues autolyzed at 37° C.						
62.0	7.0	9.0	9.0	19.3	10.3	8 hrs. after death. Autolyzed at 37° C. for 2 hrs.
—	—	8.0	8.5	12.7	11.3	14 hrs. later. Autolyzed at 37° C. for 4 hrs.

SUMMARY AND CONCLUSIONS.

1. The action of indophenoloxydase is generally diminished in the tissues (liver, renal cortex, heart muscle) of rabbits that die of pneumococcus septicemia. The diminution is more frequent and marked in the liver and kidney than in the heart muscle.

2. The diminished activity of indophenoloxydase becomes more evident when the tissues undergo autolysis. At 37° C. this ferment is much more easily destroyed in the tissues of the infected animals than in those from normal control animals (tables I, II, III, and IV). Exceptions are rare and they occur chiefly with the heart muscle.

3. Normal rabbit tissues, inoculated *in vitro* with pneumococcus culture, do not lose their indophenoloxydase much more quickly than do the normal controls, when they are kept either at room temperature or at 37° C. (tables IV, V, VI, and VII).

4. It is therefore probable that the diminished activity of the indophenoloxydase of tissues from rabbits with pneumococcus septicemia is not due to the presence of the pneumococcus in these tissues, but that it is associated with a pathological change in the animal cell during life.

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A COMPARATIVE STUDY OF THE RATE OF PROTEOLYSIS OF TISSUES OBTAINED FROM RABBITS INFECTED WITH PNEUMOCOCCI AND OF TISSUES FROM NORMAL RABBITS.*

BY FLORENTIN MEDIGRECEANU, M.D.

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In the previous paper[1] it has been shown that the activity of indophenoloxydase undergoes a diminution in the parenchymatous tissues, especially the liver and kidney, of rabbits that died of a general infection with pneumococci. This ferment is more quickly destroyed during autolytic processes in the organs from rabbits infected with pneumococci than in those from normal control animals. The presence of the pneumococcus was excluded as being responsible for this difference. It was thought that a more rapid breaking down of the nitrogenous bodies, the proteins, might be associated with the increased lability of the indophenoloxydase in the tissues of the infected rabbits.

In order to obtain definite information on the rate of proteolysis in the tissues studied (liver and kidney), the following experiments have been made. Normal rabbits, weighing 2,000 to 2,500 grams, were given intravenously 0.2 to 0.5 of a cubic centimeter of pneumococcus broth culture. The experiments have been divided into two series, those dealing with non-perfused organs and those with perfused organs.

In the first series the organs were simply removed from the body after the animal's death. In the second series the animals were killed by a blow on the neck when the clinical symptoms were severe and when pneumococci began to appear in the circulation. Im-

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* Received for publication, January 15, 1914.

[1] Medigreceanu, F., *Jour. Exper. Med.*, 1914, xix, 303.

mediately after the animals were killed they were perfused through the thoracic aorta with 0.8 per cent. saline solution. The perfusion lasted for about half an hour in each case, until the liquid running out became clear and the organs looked pale.

Normal control rabbits were killed in the same way and at the same time as the infected rabbits, or were killed when the infected animals died. All subsequent manipulations of normal and pathological material were carried out in the same manner.

The organs, after removal from the body, were thoroughly ground with half their weight of pure sterilized sand, diluted with their weight of 0.8 per cent. saline solution, and the mixture was centrifugalized; the precipitate was washed and again centrifugalized. The joined liquids were finally diluted with 0.8 per cent. saline solution so as to have a concentration in 100 cubic centimeters of about 0.3 to 0.5 of a gram of total nitrogen for the liver and of about 0.2 of a gram of total nitrogen for the kidney. Portions were taken for the determination of the total nitrogen, the total filterable nitrogen, and the amino nitrogen. The solutions, covered with a thick layer of toluol in narrow cylinders of the same diameter and thickness, were then submitted to autolysis at 37° C. During the course of autolysis, portions were withdrawn from time to time and the total filterable nitrogen and the amino nitrogen content were estimated. The total nitrogen was estimated according to the Kjeldahl-Gunning method, the filterable nitrogen by precipitating the coagulable proteins by heat and acetic acid, the amino nitrogen by Van Slyke's method.[2]

The filterable nitrogen was estimated to obtain information with regard to the disintegration of the complex protein molecules. The amino nitrogen should afford further knowledge concerning the extent of autolysis of the lower proteinic products, the peptones and the free amino acids. The results are shown in the protocols that follow.

[2] Van Slyke, D. D., *Jour. Biol. Chem.*, 1912, xii, 275.

SERIES A.

Non-Perfused Organs.

Liver.

Experiment I.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[3]				Filterable nitrogen after autolysis.[4]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		Total nitrogen		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. n 100 c.c.	Per cent. of total filterable nitrogen.	
				Liver from normal rabbit									
0.3440	0.0888	25.8	0.0348	39.2	0.1370	39.8	0.0782	57.0	0.2020	58.7	0.1300	64.4	Infected rabbit died 18 hrs. after inoculation. Pneumococci present in blood. Normal rabbit killed when infected animal died.
				Liver from pneumococcus rabbit									
0.3600	0.0938	26.0	0.0376	40.0	0.1790	49.7	0.1132	63.2	0.2450	68.1	.1795	73.2	Ten hours elapsed between death of animals and beginning of autolysis.

Experiment II.

				Liver from normal rabbit									
0.4800	0.1800	37.5	0.0537	29.7	0.2330	48.5	0.1008	43.2	0.3300	68.7	0.210	63.6	Death of infected animal 33½ hrs. after inoculation. Pneumococci present in blood. Normal rabbit killed at same time.
				Liver from pneumococcus rabbit									
0.416	0.1400	33.6	0.0514	36.7	0.2560	61.5	0.1521	59.4	0.2859	68.0	0.1714	60.0	16 hrs. elapsed between death of animals and beginning of autolysis.

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[3] Autolysis at 37° C. lasted 19 hours in experiment I, and 20 hours in experiment II.

[4] Autolysis at 37° C. lasted 62 hours in experiment I, and 160 hours in experiment II.

Experiment III.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[5]				Filterable nitrogen after autolysis.[6]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
				Liver from normal rabbit									
0.4000	0.1160	29.0	0.0431	37.1	0.2224	55.6	0.1395	62.0	0.2688	67.1	0.1822	67.8	Infected rabbit died 40 hrs. after inoculation. Pneumococci present in blood. Normal rabbit was killed 2 hrs. later.
				Liver from pneumococcus rabbit									
0.2908	0.0936	32.2	0.0326	34.8	0.1760	60.5	0.1080	61.3	0.1832	63.0	0.1082	59.1	13 and 15 hrs. respectively elapsed between death of animals and beginning of autolysis.

Experiment IV.

				Liver from normal rabbit									
0.3930	0.1210	30.8	0.0413	34.1	0.1951	49.6	0.1067	54.6	0.3390	86.2	0.2028	59.9	Death of infected animal 27 hrs. after inoculation. Pneumococci present in blood. Normal rabbit killed at same time.
				Liver from pneumococcus rabbit									
0.3600	0.0868	24.1	0.0311	35.8	0.2000	55.5	0.1012	51.1	0.2000	55.5	0.1196	59.8	8 hrs. elapsed between death of animals and beginning of autolysis.

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[5] Autolysis at 37° C. lasted 33 hours in experiment III, and 23 hours in experiment IV.

[6] Autolysis at 37° C. lasted 77 hours in experiment III, and 160 hours in experiment IV.

Kidney.

Experiment I.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[7]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
Kidneys from 2 normal rabbits									
0.2320	0.0890	38.3	0.0255	28.1	0.1550	66.8	0.0695	44.8	Infected rabbits died 27 and 33½ hrs. respectively after inoculation. Pneumococci present in blood. Normal rabbits killed when infected animals died.
Kidneys from 2 pneumococcus rabbits									
0.2550	0.1130	44.3	0.0315	27.7	0.1640	64.3	0.1111	67.7	8 and 16 hrs. respectively elapsed between death of animals and beginning of autolysis.

Experiment II.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[7]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
Kidneys from 2 normal rabbits									
0.2180	0.0704	32.3	0.0228	32.4	0.1370	62.8	0.0934	68.1	Infected rabbits died 18 and 19 hrs. respectively after inoculation. Pneumococci present in blood. Normal rabbits killed when infected animals died.
Kidneys from 2 pneumococcus rabbits									
0.2590	0.0803	31.0	0.024	27.8	0.1530	59.1	0.1104	72.1	13 and 14 hrs. respectively elapsed between death of animals and beginning of autolysis.

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[7] Autolysis at 37° C. lasted for 40 hours in both experiments.

SERIES B.

Perfused Organs.

Liver.

Experiment I.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[8]				Filterable nitrogen after autolysis.[9]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
				Liver from normal rabbit									
0.2115	0.067	31.7	0.0168	25.0	0.0721	34.0	0.0406	56.3	0.1128	53.3	0.0678	60.1	Infected rabbit was perfused 23 hrs. after inoculation. Normal rabbit was perfused ½ hr. previously.
				Liver from pneumococcus rabbit									
0.3660	0.1020	27.8	0.0379	37.9	0.2230	61.0	0.1335	59.8	0.3210	87.8	0.2170	67.7	About 24 hrs. elapsed between death of animals and beginning of autolysis.

Experiment II.

				Liver from normal rabbit									
0.2566	0.0910	35.4	0.0249	27.3	0.1124	43.9	0.0579	51.5	0.1463	56.9	0.0898	61.3	Infected rabbit was perfused 23 hrs. after inoculation. Normal rabbit was perfused ½ hr. previously.
				Liver from pneumococcus rabbit									
0.3020	0.063	20.8	0.0249	39.5	0.1520	50.3	0.0935	61.5	0.2510	83.1	0.1576	62.8	About 24 hrs. elapsed between death of animals and beginning of autolysis.

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[8] Autolysis at 37° C. lasted 20 hours in both experiments.

[9] Autolysis at 37° C. lasted 64 hours in both experiments.

Experiment III.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[10]				Filterable nitrogen after autolysis.[11]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
				Liver from normal rabbit									
0.5126	0.1210	23.6	0.0450	37.1	0.3100	60.5	0.1925	57.1	0.3650	71.2	0.2915	79.8	Infected rabbit was perfused 24 hrs. after inoculation. Normal rabbit was perfused ½ hr. previously.
				Liver from pneumococcus rabbit									
0.2844	0.060	21.0	0.0271	45.2	0.1430	50.2	0.0885	61.8	0.2020	71.0	0.1292	63.9	About 4 hrs. elapsed between death of animals and beginning of autolysis.

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[10] Autolysis at 37° C. lasted 19 hours.

[11] Autolysis at 37° C. lasted 62 hours.

Kidney.

Experiment I.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[12]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
			Kidneys from 2 normal rabbits						
0.2120	0.080	37.7	0.0303	37.7	0.1530	72.1	0.0937	61.2	Infected rabbits were perfused 23 and 24 hrs. respectively after inoculation. Normal rabbits were perfused 1 hr. previously.
			Kidneys from 2 pneumococcus rabbits						
0.1916	0.0726	37.9	0.0260	35.8	0.1327	69.2	0.0792	59.7	About 24 hrs. elapsed between death of animals and beginning of autolysis.

Experiment II.

Total original nitrogen, gm. in 100 c.c.	Filterable nitrogen before autolysis.				Filterable nitrogen after autolysis.[12]				Remarks.
	Total nitrogen.		Amino nitrogen.		Total nitrogen.		Amino nitrogen.		
	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	Gm. in 100 c.c.	Per cent. of total original nitrogen.	Gm. in 100 c.c.	Per cent. of total filterable nitrogen.	
			Kidneys from 2 normal rabbits						
0.2687	0.0978	36.3	0.0334	34.2	0.1920	71.4	0.1480	77.1	Infected rabbits were perfused 24 and 25 hrs. respectively after inoculation. Normal rabbits were perfused 1 hr. previously.
			Kidneys from 2 pneumococcus rabbits						
0.1724	0.060	34.8	0.0189	31.5	0.1310	76.1	0.0930	71.2	About 7 hrs. elapsed between death of animals and beginning of autolysis.

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[12] Autolysis lasted for 40 hours in both experiments.

SUMMARY AND CONCLUSIONS.

Liver.—The amount of filterable nitrogen in the samples before autolysis is too variable, both in the normal livers and in the livers from pneumococcus infections in the rabbit, for a constant difference to be obtained.

The amino nitrogen before autolysis is generally slightly increased in the livers of the infected animals, especially in the perfused specimens.

Most of the experiments show an increased disintegration of the livers of the infected animals during the first stages of proteolysis (twenty to thirty hours), both in the non-perfused and the perfused specimens. This increased disintegration is generally more evident for the complex proteins (filterable nitrogen) than for the lower products (amino nitrogen).

In the later stages the proteolysis of the blood-containing livers of the normal and the infected rabbits tends to an equalization, or the relations reverse, the proteolysis of the normal samples becoming more advanced. In the perfused specimens the differences in the rate of proteolysis are the same as in the earlier stages.

Kidney.—The rate of proteolysis shows no constant differences in the normal and infected tissues.

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CLINICAL OBSERVATIONS ON NINETY CASES OF ACUTE EPIDEMIC POLIOMYELITIS.

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In June, 1912, Peabody, Draper, and Dochez[1] published a report on 71 cases of acute epidemic poliomyelitis admitted to the Hospital of the Rockefeller Institute for Medical Research during the summer of 1911. During the summer and autumn months of 1912 and 1913, 90 cases were admitted to the Hospital, 57 in 1912, and 33 in 1913. The present paper reports the clinical observations made on the 90 cases admitted in 1912 and 1913, that seem to have importance from the fact that attention has not been drawn to them in earlier clinical accounts.

Cases were admitted during the acute stages only, and, after having remained in the hospital for from a few days to three months, they have reported at intervals since for observation and advice as to treatment. A far larger number were seen in the dispensary and were not admitted, as the acute stages were passed. In addition to the 90 cases admitted during 1912 and 1913, 7 cases were admitted on the possibility of their condition being due to acute poliomyelitis. The steady decrease in the number of admissions was due to a decrease in the number of cases of the disease in New York, and in addition to the decrease in numbers, a decrease in severity was observed, for in the 71 cases admitted during 1911, Peabody, Draper, and Dochez reported a mortality of 14 per cent., while among the 57 cases admitted during 1912, the mortality was 16 per cent., and among the 33 admitted during 1913 it was 9 per cent. Furthermore, the 3 fatal cases of 1913 died of secondary inflammatory conditions of the lungs, and not directly from the disease. The resulting paralyses, however, were as widespread and complete in 1913 as in the previous years.

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[1] A Clinical Study of Acute Poliomyelitis. Monograph No. 4, June, 1912. The Rockefeller Institute for Medical Research.

ETIOLOGY.

The ages of the patients varied from nine months to fourteen years. In three instances two members of a family were admitted, and in a fourth instance two cousins living in the same house. It was further observed that although only in a very few instances could a direct or indirect contact with a patient suffering from the disease be recorded, at any one time the majority of patients came from the same general locality in New York. Inquiries were made as to the prevalence of flies and other insects in the house, and subsequent to the communication of Rosenau[2] on the conveyance of infection by the stable fly (Stomoxys calcitrans), the proximity of the home to stables was in all cases inquired into. Neither the prevalence of insects nor the neighborhood of stables was found to be a usual occurrence. In many cases the parents suggested some slight accident or some dietary indiscretion as the cause of the condition, but in no case could a predisposing cause be ascertained.

In view of the possible importance of the nose and throat as the point of entrance of the infection, the tonsils were in all cases inspected.[3] Simple inspection is unsatisfactory in estimating the size or condition of tonsils, but in 37 cases they were noted as somewhat enlarged and congested, in 18 cases as enlarged, and in 4 as slightly congested. In the remaining 31 they were noted as normal or were not examined, as occurred in two rapidly fatal cases. In the case of 8 children whose tonsils were noted as enlarged and congested, 21 other children in their families were examined, and of these in 16 the tonsils were noted as showing no enlargement or congestion. In 15 cases the tonsils were enucleated, and on removal usually proved to be larger than inspection had led one to expect.

SYMPTOMATOLOGY.

1. *Preparalytic and General.*—While no cases were admitted in the preparalytic stages, 5 abortive cases were admitted, and in 22

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[2] International Congress of Hygiene and Demography, Washington, 1912.

[3] Leiner and Weisner, Wien. klin. Woch., 1910, xxiii, p. 323; Flexner, S., The Contribution of Experimental to Human Poliomyelitis, Jour. Amer. Med. Assoc., 1910, lv, pp. 1105–1113.

cases the paralysis increased after admission to the hospital. The preparalytic and general symptoms were in all cases inquired into, and are important in view of early diagnosis. These symptoms were observed by the parents or attendants for from a few hours to nine days before the onset of paralysis, but all of them were further observed in the cases after admission to the hospital and might continue until the acute general symptoms passed off. The most common symptom was *feverishness,* and this was reported to be present in 79 of the cases. It was not constant, but might appear for a day or two and then disappear before the onset of paralysis, or it might appear only after the onset of paralysis. In the records of the temperature (rectal) after admission a high temperature was exceptional, and while one case was admitted with a temperature of 104.8°, and in the fatal cases temperatures of 104° to 105° were observed before death, a temperature that varied from 101° to 103° and gradually settled in the course of a few days was seen in the majority of cases. The temperature may be settling some days before the cessation or spread of paralysis, but it was usually still slightly elevated for some days after further spread of the paralyses was observed. A child, aged three years, was readmitted for observation four months after the onset, and at that time an afternoon temperature of 100° or over was of daily occurrence, although there was no evidence of a recurrence or of any other cause of an elevation of temperature. The pulse rate did not, as a rule, settle until some days after the temperature was normal, and in a few cases showed the irregularities of sinus arrhythmia. The pulse rate was commonly between 120 and 140 on the day of admission. *Drowsiness or heaviness* was noted in 47 cases, and this was very variable in degree, for sometimes an apparently comatose condition was present, from which, however, the child could be readily aroused. Accompanying this heaviness there was a condition of *irritability or restlessness,* best seen when the child was aroused, and frequently connected in the older children with fear of pain consequent on handling. The restlessness was also observable during sleep, the child tossing about and rolling the head from side to side. In spite of the drowsiness the children seldom slept well, being wakeful and querulous, and this was in marked contrast to the sound, uninterrupted sleep seen in early convalescence. During sleep, definite involuntary *twitchings or jerkings* were seen in 20 of the patients. These jerkings were seen as short, sudden movements either of flexion or extension of a limb, and frequently in a limb that subsequently became paralyzed. The movement might involve upper and lower limbs on one side of the body, both lower or both upper limbs, or all four limbs, or head and neck, or the whole body at once. A totally paralyzed part never showed such movement, but it was most frequent in a limb that showed a slight weakness. It was not observed after the acute general symptoms passed off. A fine tremor was seen in a few of the patients similar to that described by Netter and others.[4] A lateral nystagmus was seen in one patient, and though not constant, it was present without any voluntary movement of the eyeballs. A history of a localized, profuse *sweating* was occasionally obtained, involving the part that subsequently became paralyzed, but more common was profuse general perspiration in the very severe cases. This general perspiration occurred both when the child was awake and when it was asleep, but was usually more profuse during sleep. It did not cease with the other acute symptoms or with the fall of temperature, and in one case was still well marked, though diminished, at the end of six months.

Gastro-intestinal symptoms were rarely absent, and vomiting was reported in 41 cases and loss of appetite in 22 others. Diarrhea,[5] which has been reported as common in some epidemics, was rarely given as a symptom, and was noted in only 7 cases, while constipation was a marked feature in 38 of the patients. After admission the patients in the acute stages were very thirsty, but had no appetite for solid food. When the acute symptoms disappeared, the appetite returned at once and was usually vigorous. Convulsions occurred only once, and rigors were never noted.

A symptom of considerable importance, in that it is observed early and that it is not present in gastro-intestinal disturbances, nor, as a rule, in the common infectious diseases, is the ***stiffness of neck and back,*** resistance to anterior flexion of the neck and back, or pain on attempting such flexion. This was present in 72 of the cases, and persisted for from one day after admission to three or more weeks. Of the 18 children who did not show this resistance, in 7 the posterior muscles of the neck and back were paralyzed, and the others were mild cases or were seen for the first time late in the disease. In three instances there was definite head retraction, with arching of the back, and in several more the patient was restless unless placed in a position that would allow of slight arching or retraction. This symptom was present to a marked extent in cases with only slight paralysis, and was the most marked feature in two cases classed as abortive. On the other hand, cases with extensive or severe paralysis showed slight stiffness and resistance to anterior flexion, and there was no relation between the degree of abnormality of the spinal fluid and the degree of resistance.

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[4] Paralysie infantile choreique, Arch. de Méd. des Enfants, December, 1913.

[5] Krause, P., Deut. med. Woch., 1909, xxxv, 182.

Pain and tenderness are important symptoms of the acute stage, and are sometimes observed before any paralysis is noted. Tenderness on handling was noted in 61 of the cases, and many more showed signs of pain when neck and back were flexed. In many cases the tenderness on handling was due to flexion of the neck and back, but in others it had no connection with such movement. It might be generalized and cause the child to dread the approach of anyone likely to handle him. In other cases it was localized to a limb or limbs, usually the parts paralyzed. This tenderness during the acute stages seemed to be of three types or degrees. In a few cases a hyperesthetic condition of the skin was suspected, but in dealing with patients of the age and condition under observation, accurate determination of such a point is difficult. In a few others, mild pressure on the muscle masses caused pain, not specially localized over the nerve trunks. In many more, passive movements that would stretch tendons or capsular structures caused the child to cry out with pain. The tenderness was seen frequently in limbs, that at no time showed paralysis, but the deep reflexes were usually absent. On the other hand, it could be elicited in limbs that were totally paralyzed. It frequently caused a child to keep a limb that was not paralyzed at rest and simulate paralysis, the true condition of the limb being ascertained only by handling. This tenderness, as a rule, passed off in a day or two, but might persist for three or four weeks. It constantly persisted after further spread of paralysis was noted, and passive movements increased rather than decreased its severity. After the acute stage was passed a different type of pain on passive movement was observed. If a limb were allowed to remain at rest after the acute stage, the capsular structures, tendons, and muscles appeared to stiffen, and in these cases the tenderness decreased with treatment by passive movements. This tenderness was usually localized more to the region of joints, and occurred in joints the muscles controlling which were completely flaccid as well as at joints where the muscles were only partially affected, and simulated the joint pain of an arthritis, but without local points of tenderness on pressure. Yet another type of tenderness must be noted. Some weeks after the onset a limb that has hitherto shown no sign of paralysis becomes painful on movement, the child does not use it freely, and the part appears to be paralyzed. This was well seen in a child whose left upper limb alone was paralyzed. Three weeks later, when the child was allowed to run about, she complained of pain in the knees and seemed unable to walk. On admission it was found that the muscles of both lower limbs were spastic and that the tendon reflexes were all exaggerated. The pain was situated in the flexor tendons at the back of the knees. Massage and movements relieved the stiffness, and the pain disappeared. It is possible that in such cases irritation to the pyramidal tracts results during the healing process of the lesion in the upper part of the cord.

Spontaneous pain is difficult to ascertain in patients of the age usually affected, and frequently what appeared at first to be spontaneous pain would disappear on adjusting the position of the patient to avoid tension on muscles or ligaments. In one case the pain could not be relieved by adjustment of position and seemed to be independent of such adjustments. This pain disappeared with the other acute symptoms. In this case, a girl, aged four years, there was present for two days after admission an apparent absence of sensibility to painful stimuli, as pricking with a pin caused no response, but the child was in a comatose condition at the time, and the sensibility returned as the coma passed off and the spontaneous pain manifested itself.

2. *Paralysis.*—Wickman[6] has differentiated eight types of the

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[6] Beiträge zur Kenntnis der Heine-Medinschen Krankheit, Berlin, 1907.

disease, based on the fully developed symptomatology. The distinction between these types depends on the localization of the lesions in the central nervous system. Any part of the brain and cord may be affected, and in any one case the lesions may be widespread, or lesions may be present giving at one time a paralysis of the muscles of the face and of a lower limb. The different types cannot then be rigidly differentiated, and the distinction between the meningeal and the abortive types is often extremely difficult. Müller[7] classifies all cases that do not show definite paralysis as abortive, and it is simpler to adopt his classification in this respect. Of the paralyzed cases, the differentiation of bulbospinal and cerebral types has been suggested, and though rarely cases of definite spastic paralysis are found, it is more common to find spastic and flaccid conditions coexisting.

Involvement of the *respiratory* musculature is of importance in its prognostic significance; 11 of the 12 fatal cases may be included in this group. In 7 of them death was due directly to the respiratory embarrassment, and in the remaining four it was due to secondary inflammatory conditions in the lungs. In 1 other fatal case death was due to an inflammatory condition of the lungs secondary to a paralysis of the muscles of the palate and pharynx. Of the 31 cases showing involvement of the muscles of respiration, there was paralysis or weakness of both diaphragm and intercostals in 9, and of these, 6 ended fatally while in the hospital. In 3 cases the diaphragm alone was involved without complete paralysis, and in each instance there has been complete recovery in the action of the diaphragm; 19 cases showed paralysis, complete or partial, of the intercostal muscles, and of these, 3 ended fatally and 7 recovered entirely. In no instance was the respiratory paralysis the only paretic symptom. The prognosis as to life is always grave when the respiratory musculature is weakened, and though severe paralysis of the kind is readily observed, a weakness of either diaphragm or intercostals is easily overlooked and may be the commencement of a spread that will terminate fatally. Rapidity of respiration is not necessarily present with slight weakness or limited paralysis, as either the diaphragm alone or the intercostals alone are able to carry on efficiently the movements of respiration, and even when present, the rapid, shallow respirations may be erroneously considered part of the general acute condition. If the chest is prevented from expanding by pressure on the thoracic wall, a weakened or asymmetrical action of the diaphragm can be estimated by observing or palpating its movements in descending and ascending in the abdomen, while pressure on the abdomen to limit or prevent the descent of the diaphragm will demonstrate the power of the intercostals to carry on respiration by expanding the thorax. As with any part of the body musculature, weakness of the diaphragm and intercostals may appear and disappear again in the course of a few hours.

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[7] Die Spinale Kinderlähmung, Berlin, 1910.

The large number of cases showing involvement of *facial* muscles was striking. Of the 90 cases, 31 showed weakness of the muscles of the face, and in many more a slight involvement was suspected. In 5 cases this was the only paretic symptom, while in the remaining 26 other parts of the body were involved. In 1 case, which showed many unusual factors and which will be discussed more fully later, the weakness of the facial muscles was bilateral. In 7 cases the whole of the muscles of one side of the face were paralyzed. The others, while showing a definite asymmetry, showed a weakness of the whole of one side or a paralysis or weakness of the upper or lower parts of the facial musculature only. Of the 5 uncomplicated cases, 3 showed a definite exaggeration of the tendon jerks in the upper and lower limbs, in a fourth the tendon reflexes seemed normal, and in the fifth the tendon reflexes of the upper limbs were diminished, while those of the lower limbs seemed normal. Among the cases complicated by paralysis of other parts of the body, spasticity and exaggerated reflexes were occasionally met with, but not to a greater extent than among cases showing no involvement of cranial nuclei. In the case of a girl, aged three years, admitted on the fourth day after the onset of symptoms and the third day after the appearance of paralysis, and showing involvement of the left side of the face, of the laryngeal and pharyngeal muscles, and to a slight extent of the respiratory musculature, all the tendon reflexes were exaggerated on admission. Without any paralysis of the extremities developing, the tendon reflexes gradually diminished and were lost in the course of the next two days until the only one obtainable was the right Achilles reflex, and it was but a feeble response. In 3 cases the facial weakness cleared up in the course of a few weeks, and in 5 of them no sign of facial involvement was apparent after an interval of from three to six months. At the end of that time 3 had shown no improvement, while 16 showed distinct improvement, and the remaining 4 died during the acute stage of respiratory involvement. One case deserves special notice in that an almost complete ptosis of the right upper eyelid was the only paretic symptom on admission, though when seen again six months later, slight weakness of the lower part of the face on the right side and of the right external rectus could be demonstrated, while the ptosis had almost entirely disappeared.

Difficulty with *micturition* is fairly common, and is probably due to a local rather than to a general weakness, for it occurs in cases that show slight paralysis of the limbs as well as in severely paralyzed cases and in cases that show few general symptoms. The difficulty of *defecation* is usually associated with the paralyzed or weakened muscles of the abdominal wall, but in one case the anal sphincter was observed in a patulous condition for a few days.

3. *Abortive Cases.*—Five of the cases were considered to be examples of the abortive type. In 3 no history of direct or indirect contact was obtainable, and the illness commenced with fever, vomiting, or loss of appetite and constipation, with in 2 cases general weakness and twitchings of the limbs and pains in the knees. On admission all three showed marked stiffness of the neck and back, and their spinal fluids contained 101, 34, and 9 cells respectively per cubic millimeter. The fluid with 9 cells per cubic millimeter contained a marked excess of globulin, and in all the mononuclear cells predominated. No paralyses were discoverable, though some of the tendon reflexes were exaggerated and others diminished, and nothing suggestive of any of the common acute infections in children was found. Two of them recovered entirely in the course of a few days, while the other was somewhat stiff and ataxic for four weeks. The fourth case was that of a boy, who lived in the same house with his cousin, admitted at the same time with paralysis. He was feverish and irritable at the commencement, and on admission, four

days later, showed marked head retraction, but no characteristic changes in the spinal fluid. No paralyses were observed, and he had completely recovered three weeks later. The fifth case was the brother of a child admitted two days previously with paralysis, and he was admitted after a typical history of onset, with a temperature of 103.4°, no stiffness of the neck, but with 89 cells per cubic millimeter in his spinal fluid. In the course of two weeks his spinal fluid became normal, but he remained heavy and did not recover his appetite or his spirits. No tubercle bacilli were found in his spinal fluid, but he died three months later with a rapidly terminating tuberculous meningitis. It is possible that the original diagnosis of acute poliomyelitis was incorrect in this case.

DIAGNOSIS.

In the preparalytic stages and in abortive cases the diagnosis of acute poliomyelitis is always a matter of difficulty, and until some specific bacteriological or immunological test is devised, reliance must be placed on the general symptoms and on the character of the spinal fluid.[8] The occurrence at the time of an epidemic is strong evidence in a case where the diagnosis would otherwise be doubtful. The character of the spinal fluid in other acute infections is not at present sufficiently established for much reliance to be placed on the results of lumbar puncture in ruling out such infections. In its gross characters, in the cytology and in globulin content, the spinal fluid in tuberculous meningitis and in syphilitic myelitis closely resembles that seen in acute poliomyelitis, while the clinical features of these two diseases are often indistinguishable. This is well illustrated by two cases that follow:

Case I.—L. B., a boy, aged two years and three months, had been a full-time child, difficult to bring up, but without any previous history of congenital syphilis. He was admitted with weakness of the right side of the face, right arm, and right leg, setting in after four days of drowsiness, fever, and gastro-intestinal disturbance. The paralysis was flaccid, there was no impairment of sensory functions, and there was definite stiffness of the neck. The child gave evidence of congenital syphilis in the bones of the skull, thorax, and limbs. The spinal fluid on admission contained 560 cells per cubic millimeter, mostly lymphocytes, and gave a heavy flocculent precipitate with the Noguchi test. The fever disappeared in a day or two, but the cells in the spinal fluid increased until a week after admission, when 1600 per cubic millimeter were present. The Wassermann reaction in the spinal fluid was positive, which is not found to be the case in acute poliomyelitis, and it was strongly positive in the serum also. He was transferred to the Babies' Hospital, and there the paralysis cleared up to a large extent after the intravenous administration of salvarsan.

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[8] Peabody and Draper, A Study of Cerebrospinal Fluid and Blood in Acute Poliomyelitis, Amer. Jour. Dis. of Children, 1912, iii, p. 153. Fraser, F. R., A Study of the Cerebrospinal Fluid in Acute Poliomyelitis, Jour. Exper. Med., 1913, xviii, p. 242.

Case II.—W. L., a boy, aged twenty-two months, was reported to have been perfectly well until four days before admission, when he became feverish, vomited, was constipated, and his legs were paralyzed. On admission there was a flaccid paralysis of most of the muscles of the left leg. In the course of the next few days the paralysis spread to the right leg, to the face, to the external rectus of the right eye, and, to a slight extent, to both arms. On admission the spinal fluid contained 93 cells per cubic millimeter, and on the day of death, which occurred three weeks after the onset, it contained 451 per cubic millimeter, and tubercle·bacilli were found in considerable numbers. No autopsy was obtainable, but before death the clinical picture was strongly suggestive of tuberculous meningitis. The cells were mostly lymphocytes, and the globulin reaction gave a definite precipitate, and, as in the previous case on admission, the clinical aspect and the findings in the spinal fluid were very similar to those seen in acute poliomyelitis.

From the pathology of the disease it is possible that a lesion causing recognizable clinical signs may occur in any part of the brain and spinal cord. In 3 cases where unusual clinical signs were present, a diagnosis of acute poliomyelitis was made. The cases occurred during an epidemic, and no other satisfactory diagnosis was supported.

Case III.—A girl, aged nine years, was admitted after three days of slight feverishness, vomiting, and headache, with paralysis of the left side of the soft palate. The spinal fluid was normal, the child seemed quite well, and nothing else was discovered on physical examination. No diphtheria bacilli were found in the throat, and the condition of the palate cleared up in the course of two or three weeks. Serum from this patient protected a monkey from an intracerebral injection of poliomyelitis virus.

Case IV.—The case of C. W. was diagnosed as polioencephalitis. The child was a boy, twenty-one months old, and the family history was satisfactory. He had had repeated gastro-intestinal disturbance, and had been rather troublesome to bring up. Ten days before admission he was very feverish and vomited. A day or two later there was some discharge from one ear. The fever continued, and he was drowsy and irritable. Four days before admission his left arm and leg seemed weak. On admission his temperature was 100°, pulse 80, respirations 24 per minute, and while the right arm and right leg were moved freely in all directions, the left arm and left leg were held rigid and were continually twitching. In addition, there was a rapid tremor of all limbs and of all the muscles of the body that varied in fineness and rapidity from time to time and was more marked in one part of the body than in another. Over the whole of the left side, face, limbs, and trunk this twitching and tremor were more marked than in the muscles of the right side. There was a spasticity of all the muscles of the body, more marked on the left side than on the right. The deep reflexes were exaggerated on the right side and absent, from spasticity, on the left side. No disturbances of sensation were found. Lumbar puncture gave a clear fluid with 25 cells, mostly lymphocytes, per cubic millimeter, and a slight haze with the Noguchi butyric acid test for globulin. Blood count and ophthalmoscopic and otoscopic examination were negative, and a negative Wassermann reaction was found in both serum and spinal fluid. No tubercle bacilli or other organisms were found in the spinal fluid. The temperature continued to swing from 99° to 101°. Two weeks after admission the spasticity, twitching, and tremors subsided, and on leaving the hospital six weeks later he had entirely recovered, except for a slight tremor of the left foot, and occasionally of the left arm, when he was tired or excited. Since then he has remained well. The character of the tremor, the spasticity and the flexed attitude of the arm and hand, together with the fact that during sleep the affected muscles became quite relaxed, suggested a lesion in the cerebellar-rubrospinal tract. The character of the spinal fluid, the comparatively rapid recovery, and the occurrence during an epidemic suggested that the polioencephalitis might be due to the same etiological factor as acute epidemic poliomyelitis.

Case V.—J. C., a boy, aged four years and seven months, with a satisfactory family history and a good previous history, complained of pain in the mouth four days before admission, and his lips were seen to be trembling. The next day he had difficulty in swallowing and seemed feverish, and the following day was unable to articulate or to swallow. On admission his temperature was 102.4° and after reaching 105.4° two days later, settled gradually. He was semicomatose. His face was mask-like, and he lay with head retracted and legs drawn up. The muscles all over the body were rather spastic and there were frequent clonic spasms of the right side of the face, with occasional convulsive twitchings of the arms and legs. On the day after admission the spinal fluid was clear, but contained some small white flakes and 286 cells per cubic millimeter, large mononuclear cells predominating. The cells gradually decreased in number, but the spinal fluid was not noted as normal for two months later. No tubercle bacilli or other organisms were found, and the Wassermann reactions in serum and spinal fluid were at all times negative. Except for a moderate increase in the white blood corpuscles, the examination of the blood was negative. The coma continued for nine days, and then he gradually regained consciousness and the muscular rigidity passed off. The face remained mask-like, and there was a symmetrical weakness of the muscles of facial expression on both sides. The elevators of the lower jaw were nearly completely paralyzed on both sides, the tongue was motionless except for some slight power of elevating the posterior portions, and there was no power of articulation. Saliva dribbled continuously from his mouth, and he could only swallow food when it was placed well back in his pharynx. The orifice of the fauces was wide and cavernous, and the soft palate and pillars of the fauces motionless. The right arm was weaker than the left and somewhat spastic, and showed athetoid movements and incoördination. There were no disturbances of sensation. On leaving the hospital three months later the general condition had improved very much, and the tremors, spasticity, and athetoid movements of the right arm were much less marked. When seen two months later, one side of the soft palate was active while the other was still paralyzed, and four months after that the palate had recovered, and instead of the wide, gaping aperture of the fauces, there was a small aperture with the pillars of the fauces and the soft palate quite mobile. Otherwise, little definite improvement had taken place, though he could swallow better, the saliva dribbled rather less constantly, and he made more intelligible attempts at articulation. Bilateral atrophy of the tongue was noticeable.

PROGNOSIS.

Prognosis must always be guarded in the acute stage, as muscles of vital importance may become rapidly paralyzed in the course of a few hours. It is difficult to say when the danger of further spread is over, but although cases are seen where the acute symptoms disappear only to reappear with increased severity after a few days, they are exceptional. When the fever has subsided and appetite and spirits have returned, further spread is unlikely and the prognosis as to life is brighter. A few cases run an acute course, with marked general symptoms that clear up as by crisis in a few days.

Case VI.—A boy, aged three years and eight months, complained of headache and pain in his chest, and seemed feverish for three days. He appeared to recover, but a day later the same symptoms returned. On the sixth day he was worse and seemed unable to swallow. It was thought that he had diphtheria. On admission on the tenth day of his illness he was emaciated and apparently unconscious, with marked stiffness of the neck and back and a temperature of 102.9°. The spinal fluid was clear and contained 34 cells per cubic millimeter, 95 per cent. of which were mononuclears. The face seemed a little drawn to one side, the voice was feeble, and the tendon reflexes were all exaggerated. Respirations were noisy from the bubbling of the air through the accumulation of mucus in the throat, but no involvement of the respiratory musculature was observed. During the following day the temperature rose to 103.6°, but the condition seemed otherwise the same. On the second day after admission the temperature dropped to 99.8°, and the child could talk and clear his throat, and he was eager for food. A complete paralysis of the left side of the face was the only residual involvement.

Such cases are comparatively rare, and where the acute symptoms pass off gradually it is difficult to prognosticate for some days as to whether further spread will occur or not.

The prognosis as to the ultimate recovery of power in the paralyzed parts is one of still greater difficulty. As a general rule, muscles that show the power to contract at a period when all acute symptoms have cleared up and the child is apparently well except for the paralysis will not become weaker, and can, with careful treatment, regain their power to a variable extent. If the affected muscle be one of a group of similarly functionating muscles, the others of which are active, it is more likely to recover than when all the muscles of the group are completely flaccid. This is well seen in the muscles of the shoulder girdle. The deltoid is more likely to recover if the supraspinatus and infraspinatus are active than when all the scapulohumeral muscles are flaccid. The prognosis also is better for a muscle such as the biceps if the other muscles of the arm are active than when all the muscles of the limb are completely flaccid, and there is probably a destructive lesion involving the anterior horn cells of the lower cervical and upper dorsal segments of the cord on that side. It is important, therefore, that the condition of the muscles at any time be ascertained. It is not sufficient to observe whether or not a muscle is able to perform its function, for a muscular contraction may not be sufficiently strong to move the part, and yet the muscle may not be totally paralyzed. In the legs the tendons of the dorsiflexors of the ankle can often be seen to tighten on tickling the sole of the foot, and yet no movement of foot or toes occurs, and the same stimulus may be sufficient to cause a movement in the muscle mass of the quadriceps extensor that can be felt by a hand placed gently about the thigh, but that cannot be seen or recognized by a movement at the knee-joint. If the knee be bent so that the hamstring tendons are relaxed, and the sole of the foot stimulated, contraction of the hamstrings may be recognized by a tightening of the tendons that is readily felt, although no further flexion of the knee is produced. In a similar way, action of the glutei can be ascertained by pressing with the finger-tip in the region of the gluteal fold or simply by stroking the skin in the same region; of the lumbar muscles by stimulating external to the muscle mass in the angle made by the margin of the muscles and the twelfth rib; of the vertebroscapular muscles by gently stroking the skin internal to the vertebral border of the scapula; of the scapulohumeral muscles by a similar stimulation external to the axillary border of the scapula and over the supraspinous fossa, the arm being placed in a position of partial abduction; of the pectoralis major by stimulating the inner end of the anterior axillary fold with the arm in the semi-abducted position; and of the muscles of the abdominal walls by means of the abdominal and epigastric reflexes. By similar methods of examination, other muscles that appear to be paralyzed are seen to retain some power of contraction or to have regained such power.

With the object of ascertaining whether in the early stages any prognostic value could be placed on the electrical reactions of the paralyzed muscles, 11 cases were tested. With children a general anesthetic is necessary to obtain satisfactory reactions in individual muscles, and this was not considered justifiable, so that complete reactions were not obtained, as the patients soon grew restless and impatient during the examination. It was, however, possible to test one or two muscle groups satisfactorily in the quieter patients, especially in a paralyzed limb, but in no case was it attempted until all tenderness of the acute stage was passed. The earliest to be examined was tested on the tenth day after the onset of paralysis. In this case a weakened quadriceps reacting well to faradism recovered, the calf muscles apparently paralyzed gave a weak reaction to faradism, and at the end of twelve months showed no return of power, and the anterior tibials and peronei gave no contraction to faradic stimulation, and with galvanism, kathodal closing contraction was greater than anodal closing contraction, (KCC > ACC). At the end of twelve months the anterior muscles showed no return of power, but some atrophy. In another case a paralyzed tibialis anticus muscle gave no response to faradic stimulation when examined on the fourteenth day, but with galvanism ACC > KCC the response was sharp, not sluggish, as in a typical reaction of degeneration. The muscle recovered well in the course of a few weeks. Such a reaction as this was found commonly in muscles that subsequently recovered well. Typical reactions of degeneration with slow response were found in several cases where the muscles subsequently recovered to a varying extent. It is impossible to draw conclusions from the few cases tested, and it is possible that if stronger faradic stimulation had been tried than was possible in unanesthetized patients, contractions to this form of stimulation would have been obtained. It is, however, safe to conclude that muscles giving soon after onset of paralysis no reaction to strong faradic stimulation and giving with galvanic stimulation ACC > KCC may recover.

Even if a muscle is showing commencing atrophy, a partial recovery may take place and the condition of a partially atrophied but active muscle is frequently seen. The question of whether or not the bones of a paralyzed limb will grow in length as the bones of an unaffected limb is difficult to decide without a large number of cases observed over a longer period, but it seems probable that shortening may be expected in a totally flaccid limb, especially in those cases where the muscles of the limb girdle are also completely paralyzed, while no shortening will be found in a limb with only one or two muscles or muscle groups affected. The prognosis is always worse when such muscles as the deltoid and dorsiflexors of the ankle and peronei are affected, as these muscles are more resistant to treatment than any others, and even if all the muscles of a lower limb regain, at least partially, their power, it is likely that the child will always have a limp of greater or less severity. It is impossible to state a period beyond which no further return of power can be expected, especially in cases that have not received careful treatment. In one case that had received vigorous treatment since the onset a muscle hitherto showing no action began to recover at the end of two years, but in a completely paralyzed muscle recovery to any great extent can seldom be expected after a year of unsuccessful treatment.

TREATMENT.

For the purposes of treatment, acute poliomyelitis may be divided into three stages: (*a*) the acute stage of fever and general symptoms, including the period of onset and spread of the paralyses; (*b*) the stage of recovery of muscle power; (*c*) the stage where recovery in muscle power is as complete as it is going to be, and the treatment is applied to the residual condition of deformities, flail-joints, etc. In this last stage the treatment must be surgical, and will not be dealt with except in so far as indications for such treatment arise in the stage of recovery of muscle power.

(*a*) Apart from the suggestive work of Netter,[9] in attempting to control the acute stage by subdural injections of serum from recovered cases, no specific treatment has as yet been discovered that will check the process when it has commenced. When urotropin (hexamethylenamin) is administered by the mouth it may be detected in small quantities in the spinal fluid, and since it was suggested that this might have some power to destroy the virus of poliomyelitis it was hoped that urotropin might check the processes in the central nervous system.[10] Twenty-two cases were treated with urotropin by oral administration. Cases that were still advancing on admission or cases that showed marked meningitic symptoms were usually treated in this way, and the administration continued only until the acute stage was passed, but in several cases the treatment was continued much longer. Doses of 0.3 gm. three or four times a day were employed in most instances for a child of two years. In one case the administration was continued for four weeks, during which time the patient, a child of three years, had a total of 16.8 gm. In two instances hematuria developed that ceased as soon as the urotropin was discontinued. In a child of two and a half years the hematuria appeared after five days, during which 5 gm. urotropin had been administered, and in a child of one year it developed on the sixth day after the patient had received 6 gm. In no instance did this treatment appear to cut short the acute stage, and in no instance was there any evidence of more satisfactory or

[9] Compt. rend. Soc. de biol., 1911, lxx, p. 625.

[10] Crowe, Johns Hopkins Hosp. Bull., 1909, xx, p. 102. Flexner and Clark, Jour. Amer. Med. Assoc., 1911, lvi, p. 1750.

more rapid recovery in the paralyzed parts. No decision as to the efficiency of urotropin can be reached without the statistics of a large number of cases, or by means of some precise method of estimating results.

In 1912 Clark[11] published results of the action of intraspinous injections of epinephrin or adrenalin in the treatment of the experimental disease in monkeys during the acute stage, in which the progress of the paralysis was stayed at least temporarily. In 8 cases in which the paralysis was rapidly developing and in which there was extreme respiratory involvement, this line of treatment was tried. The largest dose was 3.0 c.c. of a 1 to 1000 solution injected along with an equal volume of saline and washed in with 1.0 to 2.0 c.c. more of saline, but, as a rule, doses of 1.0 c.c. or 1.5 c.c. were used. There was usually a rise of blood-pressure of from 10 to 20 mm. of Hg. systolic, without any change in the diastolic pressure; the summit of this rise occurred in from twenty to thirty minutes. The increase of pressure disappeared in from forty-five to sixty minutes. The dose was repeated in from three to six hours, and the same rise of blood-pressure was noted. In 3 cases three doses were given. In one case, twitchings of the limbs came on a few minutes after each dose, in addition to the rise of blood-pressure. In 2 cases it was thought that the respirations were easier and the diaphragm acting better after the injections, but such improvement was slight and transitory. Of the 8 cases, 5 died, but it is impossible to say that in the 3 cases that lived the cessation of progression in the paralysis was due to the injections.

Except in cases of respiratory involvement where the patient is fighting for breath, the child lies quietly in bed and makes few attempts to move, and complete rest is assured if care is taken to make it comfortable. The twitchings and jerkings described above and occasional turning of the head from side to side appear to be involuntary and occur most frequently during sleep or in the drowsy or comatose patients. If care is not taken to obtain a satisfactory posture, however, the child may become very restless. Whenever rigidity, resistance to anterior flexion, or definite retraction of back and neck are present, the child should be supported on its side or a pillow placed beneath the shoulders or back. Pressure on the limbs and trunk should be avoided, where hypersensitiveness of the skin or deeper structures is present, by means of a wire cradle to support the bedclothes or by the clothes being stretched from side to side of a crib, and with a heavy patient a water-bed adds greatly to the comfort. The hypersensitive condition of muscles, tendons, and ligaments makes any position of a limb that results in tension on these structures irksome, and it is found that the position of semiflexion is usually the most satisfactory. If an affected arm be allowed to be at the side, the shoulders and elbows are strained, but comfort may be obtained by means of a pad in the axilla to keep the arm from the side, and the position allows the elbow to be conveniently flexed. A pillow under the knees will in the same way obtain the semiflexed position of the hips and knees, but in most instances the child appears to be quite comfortable with the legs stretched out, and it seems to be in cases of partial involvement of the hamstrings and adductors that the flexed position is desired. The position of the foot is important, and in the majority of cases requires attention. The weight of the bedclothes, on the foot, and in many cases the weight of the foot alone, will cause an extension of the ankle in cases where the anterior tibial muscles and peronei are affected, and if this is allowed to take place for even a few days, shortening of the flexor tendons results and necessitates not only much pain in correcting the position, but has also a deleterious effect on the recovery of the stretched muscles. A light wire or other right-angled splint to support the foot and relieve the tension on the extensor muscles and tendons should be applied, or the foot simply supported in the desired position with pillows. In the less severe cases that do not remain at complete rest the splint is preferable. In a limb that is not completely flaccid, the muscles, tendons, and ligaments seem readily to become stiff, and no joint should be kept in one position longer than is absolutely necessary. In most cases the pain and tenderness have sufficiently subsided in a few days to allow of gentle handling, and the joints can then be carefully moved. As soon as the acute general symptoms have passed off, the joints should be exercised to their full functional movements and the pain of the stiffened structures must not be mistaken for the pain and tenderness of the acute stage, since the resulting limitation of movement will be the more difficult to correct the longer the delay. If the pain is not relieved by posture, hot applications, aspirin, or opium preparations may be necessary.

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[11] Jour. Amer. Med. Assoc., 1912, lix, p. 367. The Action of Subdural Injection of Epinephrin in Experimental Poliomyelitis.

The general treatment during this stage is similar to that of any acute infection. In the severe and possibly fatal cases with the respiratory involvement, diffusible stimulants were administered, and when the dyspnea became marked, oxygen was administered without any lasting benefit. Artificial respiration has been attempted when the respiratory musculature failed rapidly, but in all cases the heart failed before any return of power in the respiratory muscles appeared.

Defecation is sometimes difficult to regulate, and this is seen in cases that have weakness of the muscles of the abdominal wall, and also in cases that show no apparent impairment of the voluntary muscles concerned. Large doses of cascara, castor oil, or calomel, and saline purgatives have frequently to be followed by enemas before evacuation is obtained. Similar difficulty with micturition is seen, and catheterization may be necessary, but does not often continue for more than a few days.

Little trouble is experienced with diet. During the early days the patient will not eat solid food, but is thirsty and will take sufficient milk. As soon as the general symptoms pass off, the appetite returns and there is no indication for restricting the diet. A few cases require to be fed with a nasal tube because of the paralysis of the pharyngeal muscles, and cases where the tongue or palate is involved, require the care that similar conditions in other diseases necessitate.

In spite of careful treatment, cases with impairment of the muscles of respiration or of the upper respiratory passages may develop an inflammatory condition of the lungs that results in a fatal termination, without further spread of paralysis.

(*b*) The treatment during the second stage of recovery and return of muscle power must be based on the pathology of the condition so far as that can be ascertained. The acute inflammatory action is at an end, the edema is probably disappearing, the cells that will not recover are being absorbed, and those that are less severely damaged are perhaps slowly recovering. The centres supplying certain muscles or muscle groups may be entirely destroyed, while in other centres most or only a few of the anterior horn cells are intact, sufficient, perhaps, to give the muscle power of voluntary contraction or sufficient, perhaps, to contract a small portion of the muscle or give the whole a feeble stimulus, but at first unable to cause the muscle to functionate in a recognizable manner. Every stage is seen between muscles that spontaneously recover in a few days and muscles that will never regain their power. No treatment is known that can hasten the resolution process in the central nervous system, and the muscles must, therefore, be maintained in a condition as satisfactory as possible as regards nutrition, and their control by means of the central nervous system must be encouraged. Their nutrition can be most efficiently maintained by vigorous massage and by other measures, such as hot air or water-baths that stimulate the circulation in the part. The central control can probably be encouraged most efficiently by the voluntary effort to cause the muscle to contract—that is to say, by active exercises. That electrical treatment by galvanic current can do more than act as massage is doubtful, and when the faradic current can cause contraction it would appear to be only a form of involuntary exercise due to stimuli at a point distal to the anterior horn cells, from which the voluntary stimulus is to arise. Electrical treatment may be a useful adjunct to massage and exercises, but cannot replace them. The use of high-frequency currents has been advocated,[12] even in the earliest stage, in the belief that it causes a diminution of the edema in the spinal cord, but satisfactory demonstration of its efficiency is lacking.

As soon as the acute process has subsided massage must be commenced. At first it can only be gentle and for a short period, but as the nurse or attendant gains the confidence of the child, the treatment may be increased in vigor until deep kneading of the muscle masses for twenty or thirty minutes two or three times a day can be borne. Passive movements must be performed at all the joints to prevent any limitation of movement, and it is found that if these are properly carried out from the beginning there are only in exceptional circumstances any troublesome contractures. There is danger in too vigorous application of such movements, which may stretch capsular structures and loosen joints, such as the knee or the shoulder. The active exercises must depend upon the age of the child and are most successful in the very young patients if regarded as play. A patient of less than two or three years cannot be taught to perform systematic movements, but can be stimulated to perform these movements in play. Floating toys in a warm bath provide the necessary stimulus, and the water gives a useful support to weakened limbs. A ball or a bright object may be used to encourage movements of an upper limb, and similar tricks may be devised for the lower limbs. The dispositions of the patients are very variable and no two children can be treated alike. Even if no movements of the limbs are seen, the stimulus must be applied and the child helped in the movements at first. The more the child tries, the more successful the treatment. With a child of over three years the intelligence is such that more systematic movements can be employed. Definite exercises against resistance must be detailed for each muscle or muscle group affected.[13] The weight of the limb may be sufficient resistance at first or it may be too great and help must be given, or it may be too little and gradually increasing pressure against the movement must be employed. Even though no movement is effected it must in all cases be attempted. After the muscles have been loosened and warmed by massage is the best time for the exercises. Before the muscle is strong enough to raise the part, a good method is to raise it passively and then allow the limb to fall slowly by its own weight, telling the child to prevent it from falling. In this way contractions of muscles can early be made effective. The spastic and ataxic conditions occasionally met with should be treated just as the flaccid condition by massage and movements passive and active.

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[12] Frauenthal, H. W., Jour. Amer. Med. Assoc., 1913, lxi, p. 2219.

While the muscles of a limb are recovering they regain their power at different rates, and in the majority of cases are not equally paralyzed at the commencement of the recovery, so that it frequently occurs that one muscle is capable of strong contraction while the antagonizing muscle or muscle group is still feeble. Contractures of the stronger muscles and tendons and stretching of the weaker will result, and this may tend to deformities that require operative interference if not corrected early. Stretched muscles do not recover as readily as relaxed muscles, and it is therefore important to be on the watch for such conditions and to prevent the stretching and contractures. Passive movements may be sufficient if the difference in the strengths of the muscles is not great. Splints are frequently necessary. This is well seen in the case of the ankle. When the muscles of the leg are completely flaccid the weight of the foot is sufficient to cause severe drop, but if the calf muscles are active the drop will be more difficult to prevent. The foot must therefore be fixed in the position of dorsiflexion by means of a light wire or other posterior right-angled splint or by strapping with adhesive plaster as employed by Herrick.[14] It may be sufficient to have it fixed during the night only, and free to move and exercise during the day, or it may be necessary to keep it fixed day and night, the splint being removed only when massage is to be given and the part exercised. A plaster cast or other splint that cannot be removed for treatment is to be avoided. In any situation where weakened muscles are liable to be stretched the position of the limb should be altered and maintained by suitable support. The deltoid is peculiarly situated, in that the arrangement of the origin and insertion necessitates a considerable power of contraction to raise the weight of the limb. A degree of weakness that would allow of functional movement in another muscle will give the effect of a total paralysis in the deltoid, and the return of power must be considerable in the deltoid before the abduction of the arm can be effected. The arm hangs by the side and the deltoid is kept on the stretch, a condition inimical to recovery. It is difficult to obtain the support necessary short of surgical interference such as that employed by Bartow.[15] A light splint made of poroplastic, moulded to the chest, axilla, and upper arm, is effective in many cases. Supporting the arm by means of such a splint in the position of partial abduction relieves the muscle and places it in a position more advantageous for functional movement, but is unsatisfactory in that it does not relieve the drag on the capsule of the joint. Care must be taken that with active adductors and stretched capsule and deltoid the splint does not act as a fulcrum to force the head of the bone from the glenoid cavity and still further stretch the weakened structures. While the patient is still in bed, a soft pad in the axilla is sufficient support.

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[13] Wright, Boston Med. and Surg. Jour., October 24, 1912. Muscle Training in the Treatment of Poliomyelitis.

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[14] New York Academy of Medicine, Surgical Section, March, 1914. (Not yet published.)

[15] Shoulder and Arm Paralysis of Poliomyelitis, New York Med. Jour., 1913, xcvii.

Attention has frequently been drawn to the crippling results of paralysis of the deltoid muscle and to the small extent to which recovery takes place in the case of this muscle. In 30 of our cases, one or both deltoids were noted as paralyzed completely or partially. In 16 cases the condition was bilateral and in 14 one or the other muscle only was affected, and of the forty-six muscles affected, thirty-six were noted as completely paralyzed; 9 of the 30 cases died during the acute stages or were lost for observation immediately after, and of the remaining 21 cases there were 28 deltoids available for observation. Of these twenty-eight deltoids that were observed for three or four months after the onset, 15 were carefully supported from the first, and of those two only showed no improvement at the end of that period, while of the thirteen unsupported muscles, five showed no improvement at the end of the same period.

Weakness of the extensors of the ankle and the resulting drop-foot is as difficult to affect by treatment as weakness of the deltoid, and is a source of trouble not only in the disability it causes, but also that the leg is swung out and externally rotated when the child walks, to avoid the drag of the toes. This may cause tilting of the pelvis and lateral curvature of the lumbar region of the spinal column. Ambulant braces will correct the drop, but are often so heavy that the weakened limb swings as before, and the tilting and curvature again result. The question of braces is one of much practical difficulty. If a child can walk without deformities resulting from the weight of the body acting on a weakened limb, a brace is unnecessary. If the patient is unable to support the body weight properly, without causing deformities, he should be kept off his feet as much as possible, and by means of massage and exercises against resistance the muscles should be encouraged to regain their power. The muscles will continue to improve for two or three years at least. In many cases a child cannot and should not be prevented from walking before the legs are strong enough to maintain the body weight without deformities resulting, and then some support is necessary for the joint or joints involved. The heavy ambulant braces usually applied are often unnecessarily extensive, and their weight is extremely hampering, and instead of being applied only when the child walks, they are kept on constantly so that the exercises necessary for the development of the power in the muscles are impossible, and the ultimate result is not so good.

When a child with lower limbs affected has so far recovered that they can support the body weight satisfactorily, but walking is hampered because of the foot-drop, an elastic strap from the dorsum of the shoe and fixed below the knee to a garter or to some convenient garment is sufficient to support the ankle and allows of the leg being moved forward without the swinging and tilting movement. In many cases faulty movements, such as external rotation of the thigh, are developed in the earlier attempts at walking, probably because of excessive action of the external rotators over the gluteus medius and gluteus minimus and other muscles that cause internal rotation. If care is taken during the further recovery to direct the child's attention to this fault and to urge it to swing the foot straight, the tendency to external rotation as an accompaniment of flexion disappears. The older the child the more its efforts can be utilized and directed toward a satisfactory prevention of deformities and faulty movements, and it is in the early stages of recovery that such directed effort will be most efficacious. Striking contrasts are seen between the results obtained when the mother or attendant appreciates the requirements and when the directions are carried out without proper understanding of these requirements.

The muscles of limbs and trunk can be treated effectively by massage and exercises, but those of the face, pharynx, tongue, etc., can only with difficulty be reached by such methods. These muscles, however, are so situated that they are being continually exercised and massaged by the movements of the neighboring parts, and they are continually receiving stimuli for active movements, so that further treatment seems unnecessary. The muscles of the face are amenable to electric treatment, and though it is difficult to draw conclusions as to the relative efficacy of treatment, cases treated electrically do not appear to recover more rapidly than those that are let alone. Because of the fact that cases are seen showing every stage in rapidity of recovery from the muscle that regains power in a day to the muscle that regains no power and progressively atrophies, it is peculiarly difficult to judge of what effect, if any, the treatment has had, the more so since muscles that for months show no voluntary movement and commencing atrophy will even then commence to recover in cases that have received no treatment. No two series of cases are quite comparable, since epidemics vary in severity not only as to fatality, but as to localization and severity of paralysis. Treatment must, therefore, be directed along lines that follow the indications of the pathology of the disease and that are most likely to prevent crippling and deformities. The conditions vary in each case, but by following the indications to maintain the nutrition of the muscles, to encourage movement and to prevent deformities, and by carrying out such treatment energetically, carefully and logically, many of the crippling effects of the disease can be prevented.

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PNEUMOCOCCUS INFECTION AND LOBAR PNEUMONIA.*

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Pneumonia, in many respects, certainly as a cause of death, is the most important infectious disease with which we have to deal. The symptomatic treatment is difficult and of doubtful utility; there is no well-established form of specific therapy. Nevertheless, up to within a very recent time, the investigation of the real nature of the process has been slight and fragmentary.

While the association of certain kinds of bacteria with this disease has been well established, much obscurity exists with regard to the mode of infection, the relation of the bacteria to the lesions and symptoms, the nature of recovery and, above all, with regard to the possibility of prevention or of cure. It has been in the hope of helping to shed some light on these problems that my associates and I have been making some clinical and experimental studies. It will be impossible to review in this paper all the work that has been done by others, and I shall have to content myself with presenting certain points of view which have been suggested mainly by our own work at The Rockefeller Institute.

Acute lobar pneumonia seems the best characterized of the acute lung affections. It has such a clear-cut, clinical course that it is now generally considered a distinct clinical entity, and no more to be regarded simply as an infection of the lung than typhoid fever is to be considered an infection of the intestine. This, however, is a clinical point of view and it is possible that the same kinds of reaction occur in other localized pneumococcus infections as are present when the main seat of infection is the lung.

In a very large percentage of patients suffering from inflammation of the lung of the lobar type, pneumococci are present in the lesion. In isolated instances, other organisms, as *Bacillus influenzae* or *B. pneumoniae,* are found in pure culture. It has not been our purpose to consider these latter cases, but our attention has been given entirely to the group of cases in which *Diplococcus pneumoniae* is present and is apparently the etiological agent. As is well known, diplococci, which at present cannot be differentiated from the pneumococcus, may be present in pneumonia of the lobular tube (in which the clinical course is quite distinct from that in lobar pneumonia); they may also be present in other purely localized lesions in the body, entirely unassociated with any affection of the lung, and they may even be the organisms concerned in certain cases of septicemia in man without any local lesions whatever. Moreover, organisms with identical characteristics, so far as yet determined, are found with so great frequency living on the mucous membranes of the mouth and throat of perfectly healthy individuals that they may be considered normal inhabitants of the mouth and throat cavities. In the face of such facts as these, how can it be maintained that *Diplococcus pneumoniae* is the primary cause of such a well-characterized acute infectious disease as acute lobar pneumonia? In view of the present general consensus of opinion that this theory is true, one is indeed rash even to suggest the possibility that there may be another agent concerned. On the other hand, it is important that such a possibility should not be overlooked. Even though it should be shown, however, that pneumococci do not play the primary etiologic rôle in the natural infection, their association with the lesion and their frequent invasion of the blood render it evident that they play an important part in the process and probably the most important part in the outcome, just as do streptococci in certain diseases, such as small-pox and scarlet fever, of which it is generally believed that the natural infection is due to specific etiologic agents.

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* Lecture delivered before the Harvey Society, New York, Dec. 13, 1913.

Up to within a relatively short time, the most important link in the chain of evidence that pneumococci cause pneumonia, namely the reproduction of the disease in animals, was lacking. Most important studies dealing with the experimental production of acute lobar pneumonia were published in 1904 by Wadsworth.[1] By carefully balancing the general resistance of the animal with the virulence of the race of pneumococci employed and by injecting the organisms intratracheally, he was able, in a series of rabbits, to induce a diffuse exudative pneumonia like the acute lobar pneumonia seen in man.

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[1] Wadsworth: Am. Jour. Med. Sc., 1904, cxxvii, 851.

More recently Lamar and Meltzer,[2] and Wollstein and Meltzer[3] have succeeded in regularly producing a diffuse pneumonia of the lobar type in dogs, by injecting from 10 to 15 c.c. of the fluid culture directly into one bronchus through a rubber tube passed through the trachea, following the injection of the fluid by air blown through the tube, so as to force the infectious material into the finer ramifications of the bronchi. The pneumonia produced in dogs runs a more rapid course, resolution occurs earlier—in three or four days —and the mortality is much less than in pneumonia in man.

Using a similar technic, these investigators have produced diffuse lesions in the lungs of dogs with other micro-organisms. When streptococci are injected, the lesions tend to resemble more closely those seen in bronchopneumonia in man.[4] The observers lay stress on the greater tendency in this case to a leukocytic infiltration of the lung framework, and to a much-lessened formation of fibrin. These differences between the pneumonia produced by the injection of streptococci and that following the injection of pneumococci they ascribe to inherent differences in the nature of the micro-organisms concerned, and not to relative differences in virulence. By a similar method of intratracheal injection, Winternitz and Hirschfelder[5] have succeeded in producing pneumonia of a lobar type in rabbits. In these experiments also, large amounts of the culture material (4 or 5 c.c.) were injected.

From experiments which I have carried on, using the same method, it is evident that successful results in rabbits depend somewhat on the race of organisms employed. If the organisms have very slight virulence, the animals may recover without lung lesions; if they are too virulent, a septicemia quickly results and at necropsy only congestion and edema of the lungs are present.

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2. Lamar and Meltzer: Jour. Exper. Med., 1912, xv, 133.
3. Wollstein and Meltzer: Jour. Exper. Med., 1913, xvii, 353, 424.
4. Wollstein and Meltzer: Jour. Exper. Med., 1913, xviii, 548.
5. Winternitz and Hirschfelder: Jour. Exper. Med., 1912, xvii, 657.

As Wadsworth showed, the lung consolidation is probably a manifestation of the resistance of the animal to the spread of the infection. The occurrence of the diffuse lung lesion is undoubtedly dependent on the same factors which are concerned in the differences in local reaction to the injection of virulent pneumococci in different races of animals. As is well known, mice and rabbits are very susceptible to pneumococcus infection, however induced, guinea-pigs and dogs less and man possibly still less. The result of a subcutaneous injection of virulent pneumococci into a rabbit differs markedly from that seen when a similar injection is made into a guinea-pig. In the former there is very little local reaction; a rapid general invasion of the organisms takes place, and the animal dies quickly from a marked septicemia. In the latter a marked infiltration with much fibrin formation and a slowly progressive invasion of the subcutaneous tissues occur, while there is little or no general infection. That the time element plays a rôle in the formation of fibrin is well seen in the peritonitis induced in such susceptible animals as the mouse and rabbit. If an intraperitoneal injection is made of virulent pneumococci, death occurs within twenty-four hours, and in the peritoneum there is seen only a marked congestion, possibly hemorrhages, a serous exudation and usually no fibrin. If, however, the culture is less virulent and the animal lives forty-eight hours or more, there is usually considerable fibrin over the liver, and flakes of fibrin are seen throughout the cavity. The amount of fibrin increases in ratio with the length of time during which the animal is able to resist the infection.

The experiments of Wollstein and Meltzer,[4] however, tending to show that the peculiar property of stimulating the production of fibrin is possessed to a greater degree by pneumococci, whatever their virulence, than by streptococci, is of very great significance. It is difficult to understand, however, just why this property should be a factor in the production of pneumonia of a lobar rather than of a lobular type. That this peculiar property should not be the only one concerned is made evident from the fact that the pneumococcus is the oganism most frequently concerned in lobular pneumonia in children. Dochez[6] has shown that, during the acute stages of lobar pneumonia, there is an increase of fibrinogen in the blood. Nevertheless, the coagulation time of the blood is delayed, owing probably to an increase in antithrombin as well. We know of no observations which show whether or not so great an amount of fibrinogen exists in the blood in prolonged streptococcus infections in man as is present in pneumonia.

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[6] Dochez: Jour. Exper. Med., 1912, xvi, 693.

As the virulence of the race employed is of importance as regards the production of the local reaction, so also is the number of microorganisms injected, as Kline and Winternitz[7] have shown. If the number of organisms is too small, no pneumonia results. It is well known that even in very susceptible animals a considerable number of virulent organisms is usually necessary to produce infection. Little attention has been given to the question why, when a considerable number of organisms are injected together, multiplication occurs and infection results, whereas if only a few organisms be injected, they are unable to multiply. Is it because in a culture of organisms certain ones are more resistant than others to the harmful influences, or is it simply accidental that, when a large number of organisms are injected, a few have a possible chance to escape?

Gillespie[8] has carried on some experiments with pneumococci which have a bearing on this problem. We, as well as others, have long recognized that in starting a culture of pneumococci in a large amount of bouillon, a liter for instance, a much larger inoculation is necessary in order to obtain growth than if the inoculation be made into 10 c.c., or if the culture be made on a solid medium. In the latter instance, one organism will usually produce a colony. By making the inoculations on filter-paper kept constantly wet by bouillon, it was shown that growth would occur with the inoculation of as small numbers of organisms as are required on agar, and with much smaller numbers than are required to inoculate the bouillon. The differences in growth, therefore, are not dependent on differences in composition of the medium, and further experiments have shown that they are not due to differences in oxygen supply. It seems probable that for growth to occur, the bacterium must produce changes in the medium immediately surrounding it, and that when the opportunities for diffusion are great, such local changes cannot be kept sufficiently constant unless there be a considerable number of organisms in proximity. If this be the true explanation, it may have an important bearing on infection, not only with pneumococcus but with other micro-organisms as well. The presence of mucus in the smaller bronchi, for instance, might in this way favor the multiplication of micro-organisms and so favor infection. That very large numbers of bacteria are inoculated in the experiments of Meltzer and Winternitz probably explains to some degree why their experiments have been successful, where others have failed. A second factor in the success of this technic probably lies in the fact that considerable amounts of fluid are injected and this is blown into the terminal bronchioles. Meltzer has made the interesting suggestion that by this process the bronchioles are occluded and that in this way closed cavities are formed. It is generally recognized that as long as bacteria grow on exposed surfaces, they do no harm. It is only when the growth occurs in confined spaces that harmful results supervene.

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[7] Kline and Winternitz: Jour. Exper. Med., 1913, xviii, 50.

[8] Gillespie: Jour. Exper. Med., 1913, xviii, 584.

While these experiments on the production of pneumonia in animals are of great value in showing that lesions resembling acute lobar pneumonia in man may be caused by pneumococci, they do not directly offer an explanation of the natural infection in man. It is hardly likely that in man an overwhelming infection ever occurs with numbers of bacteria so large as those used in the experiments in dogs. The usual recourse in this dilemma is to assume that the organisms concerned in natural infection have an increased virulence, or that the resistance of the host is lowered. By virulence in micro-organisms is usually meant adaptation to growth in the tissues of the host. Since in pneumonia the organisms are cultivated from the lungs and at times from the blood, we know that they have virulence for man. We have no way of determining, however, whether or not all pneumococci growing in the mouths of healthy individuals are also virulent for man. The attempts to demonstrate conclusively that pneumococci isolated from cases of pneumonia in man are regularly of increased virulence for animals have not been successful. We have found that most of the pneumococci isolated from the blood of pneumonia patients have relatively high virulence for susceptible animals, yet while certain of these cultures, when freshly isolated, are of such virulence that 0.000001 c.c. of a bouillon culture will kill a mouse, we have also obtained cultures that required 0.5 c.c. to kill. The virulence of the race, therefore, does not seem to be the only deciding factor in the question why infection occurs, even though, as will be shown, it may be of considerable importance as regards the final outcome. It must be stated, however, that it is not certain that virulence for animals is identical with virulence for man. Usually high virulence of a given race of pneumococci for one susceptible animal, as the rabbit, indicates high virulence for another, as the mouse. Ungermann, however, has described a typical pneumococcus having high virulence for rabbits, but its virulence for mice, which was originally present, was lost. We have studied a race of pneumococci originally very virulent for mice, which, after passage through guinea-pigs, increased its virulence for these animals, but became almost avirulent for mice and rabbits.

Against the view that pneumonia arises when organisms of increased virulence reach the lung is the fact that pneumonia rarely occurs in epidemics and is very slightly contagious. There are now a number of epidemic outbreaks reported, but it must be admitted that these are of rare occurrence, and all hospital experience is against contagion as a great factor in the spread of the disease. On the other hand, the fact that in certain times and places pneumonia occurs with greatly increased severity and frequency suggests that at these times the pneumococci concerned may have acquired increased virulence. When the French attempted to build the Panama Canal, the incidence and mortality of pneumonia interfered as seriously with the work as did the occurrence of malaria and yellow fever. Even during the first years of the American occupation of Panama, the mortality from pneumonia was enormous. For its decrease there seems no adequate explanation. In South Africa, deaths from lobar pneumonia among the coolies constitute a serious menace to the continued working of the mines.

Little is known concerning diminished general resistance on the part of man to pneumococcus infection and its importance in natural infection of this disease. Clinical studies have quite conclusively demonstrated that the habitual use of alcohol increases susceptibility to infection or, at any rate, renders the subject less resistant when infection has once occurred. That exposure to cold, or especially sudden changes in temperature and chilling, play a part in infection can hardly be doubted, and there is some experimental evidence to show that animals suddenly chilled are more susceptible to infection with pneumococci than others.

The view that local changes in the lung are of importance for the occurrence of infection is interesting and suggestive. In most cases of lobar pneumonia the primary seat of infection is probably in the lung. Various writers have attempted to show that infection occurs through the blood-stream, but the evidence is not conclusive. Other localized pneumococcus infections in internal organs or cavities usually occur by extension, though this is not always demonstrable. The possibility that infection through the blood may occur in certain instances cannot be excluded. Pneumonia, as a part of a general infection, however, is generally of a lobular type.

Much has been said lately about the adaptation of certain organisms for certain tissues. In many cases, however, this adaptation is more apparent than real, and the mode and degree of infection play the larger rôle in localization.

In about 50 per cent. of the cases of pneumonia, a history of preceding coryza and cough may be obtained. In these cases it is possible that there occurs a downward infection along the mucous membrane of the bronchi. The extension of the lesion through the lung from one lobe to another apparently takes place through the bronchi, as the study of large sections through lobes with beginning involvement shows. In the remaining half of the cases, however, the onset is sharp and sudden without history of bronchial involvement. Even in these cases some local change probably precedes the real infection. It is well known that thoracic trauma is frequently followed by pneumonia. The idea of Meltzer that infection may be facilitated by closure of the smaller bronchioles is most suggestive. It is possible that cold or chilling may stimulate the mucous glands so that the increase of mucus may produce favorable conditions for the growth of pneumococci, which are so frequently present in the upper respiratory tract.

That the lung is the chief seat of the disease, then, is probably due to the fact that infection occurs here, and that a local lesion results and not a general infection (at least not until late in the disease), is probably due to the fact that man is highly resistant to infection with pneumococci and that the anatomical conditions here permit of an extensive inflammatory reaction which opposes the spread of the infection.

But why does infection occur at all? Why does a person contract pneumonia? There is still considerable obscurity in regard to this phase of infection, not only in pneumonia, but also in many other infectious diseases of which the etiology is well known. The obscurity and difference of opinion in regard to tuberculosis is well known. Even our views with regard to infection in diphtheria have been disturbed by new observations. We all thought the transference of the infectious agent from the infected to the healthy throat was all that was necessary for infection in this disease. The observations of Moss, Guthrie and Gelien,[9] however, that in Baltimore there are four times as many carriers of virulent diphtheria bacilli as there are cases of the disease, are most disturbing. So far as can be determined, the bacilli from carriers differ in no way from the bacilli from patients with the disease. Moreover, the incidence of the disease seems to bear no relation to these carriers. The problem of mode of infection is thus in a minor degree analogous to that in pneumonia, in which practically the entire population represent carriers of infection.

To explain the nature of infection we may say: First, there is a possibility that in pneumonia, as in diphtheria, the organisms causing infection differ inherently from those in normal throats, especially as regards adaptation or virulence for men. Definite evidence in favor of this would be most important, but at present there is none. Second, it is possible that the general resistance of patients to pneumococci is lowered, so that organisms, formerly living as harmless parasites, now invade the tissues and induce reaction. For this also we have no definite evidence. Third, the study of artificial infection in animals, as well as the course of the disease in man,

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9. Moss, Guthrie and Gelien: Tr. Fifteenth Internat. Cong. on Hyg. and Demog., Washington, 1912, iv, 156.

suggests most strongly that local changes in the respiratory tract may precede the infection with pneumococci. Whether these are due to a primary infectious agent or to changes in the tissues due to other factors cannot be decided definitely at present. Finally, it is possible that infection depends on a combination of factors, virulence of organisms and general and local resistance each playing a part. Further knowledge along these lines is absolutely essential for prevention of this disease. To formulate rules or regulations for prevention at present seems useless, except as an experiment.

THE NATURE OF THE INTOXICATION.

Whatever be the mode of onset in pneumonia, the production of the local changes in the lungs, as well as the general systemic manifestations of the disease, seems to be in some way related to the growth of pneumococci in the body. When micro-organisms grow within other multicellular organisms acting as host, the effects on the host are of two kinds: First, there is a local reaction, in which the bacteria are present in considerable numbers, as at the point of infection. Here are induced the changes spoken of as inflammation. In addition to this, however, there is practically always a reaction throughout the entire body, even when the local reaction is very mild and evanescent. These general reactions are evidenced, not only by fever and nervous disturbances on the part of the host, but even in their absence by such effects as changes in the blood, especially the leukocytes, which indicate certain effects on the blood-forming tissues.

In most cases, the exact manner in which micro-organisms stimulate the tissues in which they are growing to a reaction which is called inflammatory, is still obscure. Since, however, identical reactions may be produced with non-living chemical substances, it is generally assumed that in the case of bacteria as well, non-living chemical substances are formed as a result of the bacterial growth, which substances are in themselves harmful. Whatever may be the exact relationship of the organisms to the local lesion, it is necessary to assume that the general manifestations of infection, and especially the effects on tissues far distant from the local lesion, are the result of soluble toxic substances which circulate in the blood or lymph. Since general manifestations similar to those in pneumonia are seen in other pneumococcus infections and even in general infections in animals without local lesions, it does not seem probable that these effects in pneumonia are due to disturbances in respiration associated directly with the lung lesions. It is possible, of course, that in pneumonia the general manifestations and effects on distant tissues and organs are due to the action *in situ* of bacteria which have gained access to the circulation and have been carried to these distant parts.

Very numerous observations have been made on the occurrence of bacteria in the general circulation in acute lobar pneumonia. During the past years blood-cultures have been made on most of the cases of pneumonia coming under my observation, and the results have not led me to change the conclusions arrived at ten years ago from the study of a series of cases, namely, that pneumococci are usually found in the blood only in the more severe cases, and the presence of the pneumococcus in the blood is of ill omen. It is possible that in all cases of pneumonia an occasional bacterium may be carried into the circulation, but the demonstration of this is difficult. That this may occur, however, is not of prime importance, for the occurrence of an occasional organism could hardly explain the great degree of effect in distant tissues, as manifested by the general symptoms which we call intoxication.

The attempts to discover something of the nature of this circulating poison have been attended with much difficulty. It would seem that a more accurate knowledge of the metabolic disturbances in pneumonia might give a clue as to the nature of the intoxication. A series of studies with this object in view was undertaken.

Of late years attention has been drawn to the occurrence of functional disturbances, especially in infants, due to derangements in salt metabolism. It appeared of interest to learn whether or not specific changes in inorganic metabolism may be induced by pneumococci, which could account, in part at least, for the symptoms induced. The most striking disturbance in pneumonia is known to be the retention of chlorids, which is frequently almost complete during the acute course of the disease. Retention of chlorids to a lesser degree is known to occur in other infections, but Rowntree[10] has shown that this retention does not occur in influenzal pneumonia to nearly so marked a degree as it does in pneumococcus pneumonia. Medigreceanu[11] has carried on a series of studies of pneumonia in dogs and Peabody[12] has studied the question in cases in man. Peabody has shown that there is a retention not only of chlorin, but also of sodium and calcium while there is no retention of potassium and magnesium, but may be a loss. Further studies indicate that the retained substances are not stored in any one place, but are spread diffusely throughout the tissues.

It is not believed that these changes are specific for pneumonia, for they probably occur in other infections. They are most striking in pneumonia, since the changes between the febrile and afebrile state occur with such suddenness. It is not likely that these changes in themselves are responsible for any of the symptoms of the disease, but in view of the striking effects which have been induced by Meltzer by changing the balance in the inorganic salts in the body, this possibility must be borne in mind. We have no knowledge of the reason for these changes in pneumococcus infection.

Pneumococci are known to produce acid readily, even, as shown by Hiss, in albuminous mediums, containing no demonstrable sugar. It has therefore been suggested that the symptoms in pneumonia are the manifestations of an acidosis. Hamburger[13] has attempted to explain the chlorin retention on the basis of a febrile acidosis. The studies of Peabody,[14] however, have shown that the curves of chlorin retention and of ammonia excretion, which is generally considered the best indicator of acidosis, do not necessarily run parallel. The studies of inorganic metabolism have therefore given no conclusive insight into the nature of the intoxication.

In order, if possible, to obtain some knowledge regarding this problem by another method, Peabody has made studies of the gas exchange in the blood in pneumonia. He has found that in this disease the carbon dioxid in the venous blood is quite regularly low,

[10] Rowntree: Bull. Johns Hopkins Hosp., 1908, xix, 367.

[11] Medigreceanu: Jour. Exper. Med., 1911, xiv, 289.

[12] Peabody: Jour. Exper. Med., 1913, xvii, 71.

[13] Hamburger, H. J.: Osmotischer Druck und Ionenlehre, Berlin, 1912.

[14] Peabody: Jour. Exper. Med., 1912, xvi, 701.

in spite of the disturbances in gas exchange in the lung. At the same time there occurs an increase in the ammonia nitrogen in the urine, and the curves run somewhat parallel. These changes, which are indicative of increased acid formation, nevertheless correspond to changes that have been known to occur during fever and infection due to other causes, and are no indication of specific changes occurring in this disease. The carbon dioxid content of the blood does not bear a definite relationship to the severity of the disease, except that it is lowest in the most severe cases and in the terminal stages.

On the other hand, the study of the oxygen-content of the blood has revealed some interesting changes. Studies of the peripheral venous blood showed in certain cases a diminution in the oxygen-content of the venous blood. In studying the blood in one such case, it was found that the blood would not take up a normal amount of oxygen, and this in spite of the fact that the hemoglobin content was normal. In a careful study of such blood by Butterfield and Peabody[15] it was found that this phenomenon was due to the formation of methemoglobin. This change also occurs regularly in the blood of rabbits[16] infected with the pneumococcus and has no relation to the lung lesion. It also occurs when the bacteria are grown in blood-containing mediums.

Usually the change into methemoglobin in the animal body does not go so far that the methemoglobin can be distinguished spectroscopically. In the test-tube, however, especially when hemoglobin in solution is added to the culture, practically all the hemoglobin may be changed into methemoglobin. That this reaction is not simply due to the action of acids formed by the pneumococcus is shown by the fact that for the production of methemoglobin far more acid is required than could be present in the body, and, moreover, that it may occur in cultures or filtrates that are alkaline in reaction. It is therefore evident that this change is due to the action of a poison formed by the pneumococci.

Peabody[17] further made a study of the blood in twenty-five cases

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15. Butterfield and Peabody: Jour. Exper. Med., 1913, xvii, 587.
16. Peabody: Jour. Exper. Med., 1913, xviii, 1.
17. Peabody: Jour. Exper. Med., 1913, xviii, 7.

of pneumonia to determine the frequency of the occurrence of this phenomenon and the time of its appearance. Of the cases which ended in recovery, in only one was there any indication of a diminution of the oxygen-absorbing power of the hemoglobin. In all of the ten cases ending fatally, there occurred a progressive loss in the oxygen content of the blood and in the oxygen-combining power of the hemoglobin, and from the previous studies it is certain that these changes are due to the formation of methemoglobin. In nine of the ten cases the blood-cultures were positive.

That these changes play a part in the fatal termination can hardly be doubted. The terminal symptoms of the disease may be accounted for by deficient oxidation. It is not likely, however, that these changes in the blood are in themselves the only factor in accounting for the fatal result; but they represent one of the factors, and are an indication of the intoxication which is the result of the growth of pneumococci in the body.

A second effect of the pneumococcus intoxication has been demonstrated by Medigreceanu† by estimating the amount of oxydase in the organs of animals dying from pneumococcus septicemia, as compared with the organs of normal rabbits. In these studies Medigreceanu employed the method of Röhman and Spitzer, which is based on the property of tissues of oxidizing a mixture of naphthol and paraphenylendiamin into phenol. By comparing the tissues of normal animals with those previously infected with pneumococci, it has been found that this oxydase is generally diminished in the latter animals. By proper controls it has been possible to show that this change is due, not to the presence of pneumococci in the tests, but to some change which results in the tissue from the infection. Another effect of the action of the toxin on tissue function is thus made evident. It is therefore probable that, in addition to the lessened supply of oxygen by the blood due to the formation of methemoglobin, there is also a lessened power of the tissues to carry on the proper oxidation function.

Finally, in order to obtain evidence of the presence of a poison, studies were made by Medigreceanu[18] to determine whether or not there was an increased output of substances known to have the property of neutralizing poisons arising in the body. Such a substance is glycuronic acid, and it was found that during the acute stages of pneumonia in man, in almost all cases, there is a definite increase in the output of this substance.

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† Medigreceanu: Jour. Exper. Med., 1914, xix, 309.

[18] Medigreceanu: Jour. Exper. Med., 1913, xviii, 259.

All these studies clearly indicate the activity in pneumonia of a circulating poison; but the direct demonstration of the presence of this toxic substance in the animal is more difficult. To this end the following experiments were performed. Each one of a series of rabbits was inoculated with an overwhelming dose of pneumococci. Then, just as death was imminent in from five to eight hours, the animal was bled to death, and as quickly as possible the blood was defibrinated, the serum passed through a Berkefeld filter to remove the bacteria and the filtrate injected intravenously into a normal rabbit. To our surprise and disappointment, the animals did not die, nor in a second series of rabbits treated in this way were we able to detect any minor harmful effects of such injections.

When one considers the conditions in pneumococcus infection it is not surprising that there is great difficulty in demonstrating the presence of toxin in the animal, or even of demonstrating the production of toxin by the pneumococcus *in vitro*. The infectious diseases in which active specific toxins have been well demonstrated *in vitro* are diphtheria and tetanus. In these diseases, however, the conditions are unusual. Here a moderate number of organisms growing in the local lesion produce sufficient poison to bring about the most profound intoxication, and it is not surprising that the poison may readily be demonstrated *in vivo* and *in vitro*. In pneumococcus infection, the conditions are different. Even in general infection in the highly susceptible mouse or rabbit, the number of organisms growing in the body is enormous before the animal finally succumbs.

In the severe and fatal cases in man the blood may contain as many as 65,000 organisms per cubic centimeter; and when it is conceived that these are throughout all the body-fluids and the tissues, it is evident what immense numbers of bacteria are responsible for the intoxication and fatal outcome. In man, even when there is no marked invasion of the blood, the number of micro-organisms in the lung must be very large. It is probable that during the course of the disease bacteria are all the time undergoing degeneration, so that from the beginning to the end, large numbers of bacteria have been present. Also the amount of toxin present at any one time may be very small, yet when acting on tissue-cells for six or seven days may produce marked effects.

The suggestion has been made that in pneumonia the symptoms are due to the absorption of products of digestion of the pathological exudate in the lung. It has been well established by various observers that, during the parenteral digestion of protein, substances are formed which may induce fever and symptoms of intoxication. Similar symptoms may be induced by the injection of peptone and other products of protein digestion, into the circulation of animals. Most of the work that has been done in the production of fever by means of protein, however, has been carried out with foreign protein and not with the protein of the host. Moreover, at the time the resolution is probably greatest, that is, following the crisis, such symptoms are not present, although in most cases considerable amounts of the digested exudate are being absorbed. It is hardly likely that the specific and characteristic symptoms of pneumonia can be due merely to the absorption of the products of digestion in the local lesions.

Following the discovery of serum anaphylaxis in guinea-pigs and its relation to protein intoxication in man, numerous efforts have been made to bring this into relation to the toxic effects seen in acute infectious disease. Friedberger has shown that if bacteria be treated with immune serum and then with complement, the supernatant fluid, after removal of the bacteria by centrifugalization, will be toxic when injected into a guinea-pig, the animal dying within a few minutes with symptoms identical with those seen in anaphylactic shock. He has called the toxic substance produced outside the body in the manner stated "anaphylatoxin," and believes that it is identical with the substance formed within the body which produces the symptoms following the second injection of protein. He thinks that this experiment may offer the explanation for all bacterial intoxications. According to his theory in the period of incubation, during which the bacteria are already present but produce no symptoms, antibodies are being formed, and when these are present in sufficient numbers, the bacterial bodies begin to be split up and the substances so formed produce, not the acute symptoms which are speedily followed by death, because the bacteria are not present in sufficient numbers, but milder symptoms—fever, etc., a kind of chronic anaphylaxis. This can hardly explain, however, what occurs in pneumonia, in which all the evidence seems opposed to a long incubation period, the onset of the symptoms being sudden and apparently the immediate result of the infection.

The production of the so-called "anaphylatoxin" from pneumococci may readily be done, as we and also Neufeld and Dold have shown. Neufeld and Dold,[19] moreover, have shown that similar toxic substances may be obtained from bacteria by simple extraction in salt solution containing lecithin. Rosenow[20] then showed that if pneumococci are merely placed in salt solution for forty-eight hours at 37 C. (98.6 F.), the extract so formed is toxic, and on injection intravenously into guinea-pigs, acute symptoms and speedy death, like those seen in serum anaphylaxis, result. We have studied the effect of the injection of extracts obtained by autolysis in a very large number of guinea-pigs,[21] and, in our experience, while occasionally sudden death is produced, this does not occur with great regularity.

Since the salt solution extracts of pneumococci did not show as high toxicity as was anticipated, it was held possible that in the peritoneal cavity of an animal the solution of the bacteria might go on at a more rapid rate, from which cavity solutions might be obtained of greater and more constant toxicity. Guinea-pigs were therefore inoculated intraperitoneally with large doses of pneumococci. As soon as possible after death, the peritoneal cavity was washed out with salt solution. The cells and bacteria were then removed from this solution by centrifugalization and the supernatant fluid was used for intravenous injection into healthy guinea-pigs. Of eleven animals so treated, eight showed immediate symptoms like those seen in anaphylaxis, and four of these died within a few minutes with typical features of anaphylactic death and with characteristic necropsy findings.

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[19] Neufeld and Dold: Berl. klin. Wchnschr., 1911, xlviii, 1069.

[20] Rosenow: Jour. Infect. Dis., 1911, ix, 190.

[21] Cole, Rufus: Jour. Exper. Med., 1912, xvi, 644.

From the experiments it is evident that the development of the toxic substance is more constant and striking in the peritoneal cavity of the guinea-pig than it is in the test-tube. In the animal body, however, conditions are complex and it is difficult to know whether the toxic substance is specific or bears any direct relation to the infectious agent. We therefore tried to obtain solutions of the pneumococcus bodies by other means. Making extracts in chloroform and in ether did not yield solutions that could be readily studied. We next studied the solution of pneumococci obtained by means of bile. In making the solutions a 2 per cent. solution of sodium cholate in normal salt solution was employed. The effect of the intravenous injection of bile extracts of pneumococci has now been tested in a very large number of guinea-pigs and rabbits. In a large proportion of cases death with acute symptoms resembling those in anaphylactic shock occurs. When smaller amounts of the extract are injected, or when the toxicity of the extract is less, the animals die in from two to twelve hours. Such animals usually show more or less pulmonary edema and hemorrhages, and small hemorrhages are present in the peritoneum and diaphragm and in the walls of the stomach and intestines.

It is probable, from the effects produced, that the substances operative here are the ones that produce the effects in "anaphylatoxin" and in the salt solution extracts. In the latter case it has been assumed by Rosenow that the toxic substances result from the digestion of the bacterial protein by the ferments contained in the bacterial cell. The proof of this, however, does not seem convincing. The fact that the solution of the pneumococci in cholate solutions may occur within one-half hour at 4 C. (39.2 F.) is evidence that in this case the active substance is not the result of ferment action. In a recent communication Jobling and Strouse[22] have presented good evidence to show that the lysis of pneumococci in salt solution is probably not merely the result of ferment action. All these experiments indicate that the bodies of pneumococci contain substances which are toxic when they are set free by the solution of the bacterial bodies. They therefore present evidence in favor of the well-known endotoxin theory of Pfeiffer.

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[22] Jobling and Strouse: Jour. Exper. Med., 1913, xviii, 597.

During the past few years this theory of the origin of toxic substances has been largely neglected, owing to the interest in the theories of Vaughn and Friedberger, according to which the intoxication in all forms of infection is caused by substances which are intermediate products in the digestion of protein. Vaughn goes so far as to state that the substances producing the symptoms are identical in all infections and that the different symptom-complexes are dependent, not on the nature of the intoxicating substance, but on other conditions. It would hardly seem, however, that the intoxicating substance causing the rapid pulse and rapid, labored respiration and violent delirium of pneumonia is identical with the intoxicating substance in typhoid in which there is a relative slowing of the heart and low, muttering delirium. Though the intoxication may be due to the products of protein digestion, it does not necessarily follow that the substance is the same in all cases, as the bacterial proteins must differ enormously in composition.

While the obtaining of toxic substances from the bodies of pneumococci is of great interest, it is quite evident that this, in itself, does not contain the proof that we are dealing with the substances responsible for the intoxication in pneumonia. In order to present such evidence, further knowledge is required of the nature of the substance and especially of its relation to pneumococcus immunity.

Certain facts have already been established in regard to this toxin. Its study has been greatly facilitated by the fact that when added to washed sheep-corpuscles hemolysis occurs. So far as studied, the toxic effects are caused by the same substance which produces hemolysis, since the two properties are influenced by the same measures and vary in equal degree. One of the most important facts that has been determined in regard to this toxin is that the toxic and hemolytic properties vary with the virulence of the organism employed. Extracts from non-virulent cultures, so far as studied, are not toxic. The substance which is responsible for the formation of methemoglobin in the body and the discoloration of blood in cultures, however, does not seem to be present in this toxin. The toxin is destroyed by heating one-half hour at 56 C. (132.8 F.).

This may explain why the injection of pneumococci killed by heat produces no effect in the animal injected. It loses its toxicity when kept for twenty-four hours at 37 C. or for two or three days on ice. It may be dried, in which condition its toxic properties disappear much more slowly. It does not pass readily through a Berkefeld filter and it is precipitated by colloidal iron solutions. Many attempts have been made to neutralize its action by the use of dyes, by nucleic acid, nucleates, glycocoll, glycuronic acid, etc., substances which are considered to attach themselves to toxic substances in the body and thus to render them non-toxic. None of these experiments have been successful. The only substance so far found which is able to neutralize the effect of the toxin is cholesterin. When cholesterin is mixed with the toxic substance and kept at 37 C. for fifteen minutes, the toxic effect, as shown by injection into animals, and also the hemolytic effect, is lost. When the toxin is mixed with cholesterin and immediately injected into the animal, however, or when the toxin is first injected and is immediately followed by the cholesterin, the toxic effects cannot be prevented. Nor can the toxic effects be prevented by injecting the cholesterin before the toxin. The most important results in this study have been obtained in the attempts to produce antiserums to these toxins, and of these I shall speak later.

Whatever may be the mechanism by which intoxication is brought about, have we any evidence as to the determining factors in the final outcome, that is, as to why the patient recovers or dies? The results of our blood-cultures would seem to indicate that the occurrence of septicemia plays an important part in the death of the patient. A study of the virulence of the cultures from the blood also seems to show that the intoxication is greater and the prognosis worse when the organisms have a high virulence than when they have a low virulence. Moreover, our clinical experiments seem to indicate that the progressive extension of the local lesion is of bad prognostic import; that the failure of the body to erect a limiting barrier to the local extension of the disease is an important factor in the fatal outcome. At any rate, it is certainly true that in most fatal cases, on examining the lung, one sees, not a sharply localized lesion, but an extending lesion frequently involving several lobes.

This progressive extension seems to bear some relation to the virulence of the organism. With organisms of low virulence, the body is able to resist the infection, as regards both the spread of the local lesion and the general infection.

We have made quite extended studies to learn something of the nature of the general resistance of the body to the pneumococcus infection and its effects. It would seem that in pneumonia with its sudden crisis—one of the most startling and dramatic events with which the physician has to deal—an ideal opportunity would be offered to learn the nature of the process of recovery. It must be borne in mind, however, that only in certain cases does such a critical change in the patient occur. Of about 10,000 cases collected by Musser and Norris[23] the temperature fell by crisis in only about half. In the other cases it is difficult to determine with accuracy just when the change in the patient's condition occurs. It is therefore a mistake to think that in pneumonia we have a sudden change from susceptibility to resistance. More likely the process is a gradual one, and the marked change in the patient's condition occurs when the resisting factor, which increases gradually, reaches a degree sufficient to be effective. This factor of resistance may not be a single one, but the result may be due to a summation of several factors.

It has been suggested that the crisis represents a kind of anaphylactic reaction. It is known that following serum anaphylaxis there occurs a period during which the animal is in a refractory state. If the intoxication in pneumonia is due to peptone-like substances derived from the bacterial protein, it is possible that the crisis is a form of cumulative shock, following which the patient is refractory. Little is known, however, concerning such prolonged anaphylactic intoxication, and the nature of antianaphylaxis is still so obscure that it does not seem profitable to dwell longer on this theory, attractive as it is.

The fact that the crisis usually occurs in about seven days is strongly suggestive that the reaction is a true immunity reaction, since it is about in this time that antibodies appear in the blood in their maximum concentration, as we know from artificial immunization.

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[23] Musser and Norris: In Osler's Modern Medicine, Philadelphia, 1907, ii, 537.

The view that recovery in pneumonia is due to the production of immune substances presupposes that at the end of an attack of pneumonia the patient is immune. We know from experience, however, that this is not so, or if immunity is present, it is of very short duration. I have seen a patient return to the hospital with a typical attack of pneumonia two days after discharge from a previous attack. Moreover, it is well known that a person may have repeated attacks; in fact, one attack seems to render a person more susceptible. It is quite possible, however, that the relative natural immunity of man requires only a very slight assistance in the shape of acquired humoral immunity in order to render the body able to overcome the infection, and following this the immune bodies may very quickly disappear from the blood. The attempts to demonstrate the appearance of known immune substances in the blood during and following an attack of pneumonia have not previously been very successful. An increase of the ordinary bactericidal substances which act in conjunction with complement has not been proved. Most observers have found that the pneumococci grow quite well in the blood-serum of patients recovering from pneumonia, even in the serum of immunized animals.

It has been asserted that by combining the leukocytes and serum of pneumonia patients, or by using the defibrinated blood, definite differences may be demonstrated between the blood of normal persons and that of patients during or following the crisis. None of these experiments seems to me free from objections. There does not seem to be sufficient evidence for the conclusion that the recovery is due merely to an increase of opsonins, though Clough,[24] who has studied the phagocytic activity of the serum obtained after crisis or lysis in a series of eleven cases, found in six of these definite power of the serum to bring about phagocytosis of virulent pneumococci. In two of these cases the serum was tested before crisis and showed no such action. In his experiments, with one exception, the phagocytic activity was limited to the homologous strain. It has been stated by Rosenow that a difference exists as regards phagocytic activity between the leukocytes of patients with pneumonia and those of normal persons, though the results of others (Tunnicliff and Eggers) do not confirm these conclusions. Wolff[25] has attempted to show the increase of phagocytic power in the blood of pneumonia patients by making a composite curve combining the number of leukocytes with the opsonic index. We feel, however, that the errors in the usual opsonic technic are too great to justify his conclusions.

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[24] Clough: Bull. Johns Hopkins Hosp., 1913, xxiv, 295.

Of more importance are the experiments showing an increased protective power for mice of the blood of patients after recovery from pneumonia, as tested against known lethal doses of pneumococci. Neufeld has shown that while normal human serum had no protective action, that obtained from certain patients following the crisis had a definite effect. Strouse,[26] and Seligmann and Klopstock,[27] however, failed to demonstrate such changes. Studies on this question were therefore undertaken by Dochez[28] on a series of cases. The method used was the following: Specimens of the patient's serum were obtained on various days both before and following the crisis. When possible, the organism against which the serum was to be tested was cultivated from the patient, either from the blood or sputum. In case the pneumococcus, when isolated, was of low pathogenicity, the virulence was raised by successive animal passages until a dose of 0.000001 c.c. of a broth-culture was sufficient to kill. Twenty-four-hour broth-cultures fresh from animals were used for infection, and the serum and varying quantities of the culture were mixed in the barrel of a syringe and immediately injected intraperitoneally. The appearance of protective substances in the blood could then be detected, as shown by the protocol from one such experiment (Table 1).

The serums from fourteen cases of pneumonia were so studied. In ten of these the serums were tested against homologous organisms. Of these ten cases all but one showed at some time the appearance of protective substances in the blood. Of the serums

[25] Wolff: Jour. Infect. Dis., 1906, iii, 731.

[26] Strouse: Jour. Exper. Med., 1911, xiv, 109.

[27] Seligmann and Klopstock: Ztschr. f. Immunitätsforsch., Orig., 1909-10, iv, 103.

[28] Dochez: Jour. Exper. Med., 1912, xvi, 665.

TABLE I.

Protective Power of Serum of an Untreated Patient with Lobar Pneumonia at Varying Stages During the Disease.

Quantity of Culture in c c.	Quantity of Serum in c.c.	Virulence; no Serum	Control; Normal Serum	Serum Obtained								
				Three Days Before Crisis	Two Days Before Crisis	One Day Before Crisis	Three Hours After Crisis	Two Days After Crisis	Four Days After Crisis	Five Days After Crisis	Seven Days After Crisis	Eight Days After Crisis
0.1	0.2	—	—	—	24†	24†	32†	29†	42†	42†	32†	24†
0.01	0.2	—	—	—	42†	20†	75†	42†	42†	27†	—	32†
0.001	0.2	27†	42†	42†	29†	42†	66†	*	*	*	42†	42†
0.0001	0.2	42†	42†	66†	42†	66†	*	*	49†	42†	44†	42†
0.00001	0.2	50†	42†	32†	42†	42†	*	*	*	42†	66†	45†
0.000001	0.2	45†	43†	42†	47†	66†	*	*	*	*	45†	96†

* Animals protected as shown by survival.

† No. of hours before death of animal injected.

from four cases tested against stock cultures, only one showed any protective power. The amount of protection was never very high, though in some instances 0.2 c.c. of serum was able to protect against one thousand times the minimal lethal dose. The time of appearance of the protective substances varied somewhat, though in seven instances protective substances either appeared for the first time or showed a marked increase in amount at the time of crisis or, in case of lysis, during the period when the symptoms were abating. In two cases the serum taken during the period of defervescence exhibited little or no power of protection, even against homologous strains, and it was not until some time later, in one case sixteen days, that the presence of protective substances in the blood was demonstrated.

Clough[24] later carried out a similar set of experiments, and in nine out of twelve cases the serums after crisis or lysis showed definite protective power against homologous strains. The technic used differed somewhat from that employed by Dochez and the results were not so striking, but show well that in most cases the serum acquires definite protective power.

These experiments are of great importance as showing, first, that in many cases, at least, protective substances appear in the blood of patients recovering from lobar pneumonia, and second, that these protective substances in many cases are active only against the race of organism concerned in the infection. These experiments, however, do not yet establish that crisis or recovery in pneumonia is due alone to the development of these protective substances in the blood. As already stated, in certain cases they cannot be demonstrated. It is altogether probable, however, that they play some part in the final outcome. As to the nature of the substances which are most active, it is impossible at the present time to state.

Probably recovery from pneumonia occurs when the growth of the organisms is inhibited and their toxic effects neutralized. It is impossible to state which comes first. It is conceivable that if the toxic effects of the bacteria are neutralized, the body is readily able to cause their destruction, since it is possible that pathogenicity depends entirely on toxicity. There is some evidence, as I shall show, that immune serums are antitoxic. With the present technic it has not been possible to demonstrate increase of antitoxic power of the patient's serum during the crisis.

In the immune-body theory of the crisis, the local lesion is left entirely out of consideration. It is quite evident that in pneumonia we are dealing, not merely with a septicemia, but with a condition in the lung which has a very important bearing on the termination. The involved portion of the lung forms a solid mass in which are growing numbers of micro-organisms. In each alveolus are fibrin, leukocytes, red blood-corpuscles and bacteria, and in the spaces free serum. Now it is known that as the process advances, the number of leukocytes becomes greater and greater. Resolution finally occurs almost certainly as a result of this increase and associated breaking down of the leukocytes, and with this the setting free of ferments which bring the fibrin into solution. The fact that this does not occur earlier is due to the overbalancing of the leukocytic ferments by the antiferments of the serum, and the lytic ferments become active only when the relation between leukocytes and serum is in favor of the former. It is conceivable that recovery only ensues when such a balance occurs and when, with the solution of the fibrin, tension is relieved and there is an outlet for the exudate. Instead of the surgeon inserting a knife, nature does the work by injecting a ferment.

It is quite probable, moreover, that during resolution other factors than the purely mechanical are at work. With the solution of the exudate, numerous substances are formed which have a direct destructive action on the bacteria. Such substances as the soaps of fatty acids, which are known to have such a destructive action, have been demonstrated in the resolving lung by Lamar.[29] Moreover, it is well known that during the growth of pneumococci outside the body, substances are formed in the culture medium which themselves are destructive. It is quite probable that such substances are being formed in the lung and they may aid in bringing about destruction of the pneumococci. Against the view that crisis depends mainly on resolution of the exudate, however, may be brought the very evident and conclusive objection that they do not necessarily occur synchronously. Resolution may be long delayed, or resolution may be occurring in one part of the lung while the process is advancing in another.

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[29] Lamar: Jour. Exper. Med., 1911, xiii, 1.

That leukocytes play some part in recovery is rendered probable by the experiments of Klein and Winternitz.[30] They have shown that when rabbits are treated with benzene, a leukotoxic substance, the animals rapidly succumb to pneumococcus infection, whereas when they are treated with toluene, which is a similar substance but which has no effect on the leukocytes, no decreased resistance is seen. Whether the chief function of the leukocytes consists in limiting the local infection, in which they undoubtedly play a rôle, or in aiding in the development of a general immunity is not indicated by these experiments. Clinicians, however, have long been of the opinion that a low leukocyte content of the blood is unfavorable.

It is not unlikely that in recovery all of the factors mentioned play a part. The destruction of the bacteria in the lung lesion may depend on local factors quite different from those responsible for the destruction of the bacteria in the circulating blood. From present knowledge it would appear that the growth of bacteria in the blood is the most serious part of the pneumonic process, and it seems that this, at least, is influenced by the appearance of circulating anti-bacterial substances.

METHODS OF CURE.

It has been known since 1891 that susceptible animals may be rendered resistant to the action of pneumococci by the injection of increasing and properly spaced doses of pneumococci, beginning with the dead organisms. Moreover, it was early shown that if a very small amount of the serum of the immunized animal is injected into a second animal, this animal for a short time is also immune. These experiments are so striking and fundamental that it is no wonder that various attempts have been made to prepare and use such serums therapeutically. The clinical results, however, have not been convincing. Certain observations made principally by Neufeld and his collaborators and other observations made in our own laboratory, suggest reasons why such results have not been satisfactory and methods for overcoming the difficulties.

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[30] Klein and Winternitz: Jour. Exper. Med., 1913, xviii, 50.

Opinions have differed as to whether or not an immune serum produced by the injection of a given race of pneumococci into an animal is effective against all races of pneumococci. The first accurate studies on this problem were made by Neufeld and Händel.[31] They tested a so-called univalent serum against various races of pneumococci. While this univalent serum was protective in mouse experiments against fifteen strains studied by them, against other strains the serum had practically no effect. They decided that these atypical strains were not *Serum-fest* in the ordinary sense of the term, since the serum obtained during convalescence from one of the patients, from whom one of these organisms had been isolated, protected mice against the homologous strain and also against one of the other atypical strains, but did not protect against the typical strain. They then produced an immune serum against one of the atypical strains to see whether all atypical strains could be affected by this immune serum, but found this not to be true. In their further studies they found that the second immune serum, which they called *Serum Franz*, protected against only three of the atypical strains isolated by them, but failed to protect against three other strains. These latter three strains they further showed to be individual in their reactions. Neufeld and Händel did not have access to a large number of patients with pneumonia from whom to obtain cultures, and could not determine the frequency of occurrence of atypical types, nor could they make extended studies on grouping of the organisms on a biological basis, though from their studies the possibility of making such a grouping was most evident.

With the opening of the Hospital of The Rockefeller Institute in October, 1910, patients suffering from lobar pneumonia were admitted for treatment and study, and an extended study was commenced of the pneumococci obtained in these cases. An immune serum was prepared by injecting a horse with a culture of pneumococcus obtained from Professor Neufeld, the same race he had employed in the production of his immune serum. The protective power of this serum for mice was tested against a number of races

[31] Neufeld and Händel: Ztschr. f. Immunitätsforsch., 1909, iii, 159; Arb. a. d. k. Gsndhtsamte, 1910, xxxiv, 169; ibid., 1910, xxxiv, 293; Berl. klin. Wchnschr., 1912, xlix, 680.

of pneumococci cultivated from a series of cases of pneumonia. A report by Dochez[32] gave a preliminary report of this study, indicating that this serum protected against only about half the races studied. It was therefore evident that if such a serum were employed therapeutically, an effect could be expected at the most in only about half of the cases treated.

Experiments[33] were then undertaken to determine whether it would be possible to make a biologic classification of pneumococci obtained from cases of pneumonia, based on their reaction to different serums in protection experiments. Rabbits were therefore immunized to each of the races which were not acted on by the horse-serum, which we have called Serum 1, and the protection afforded by these different rabbit serums against all the other races tested. A considerable number were found to show cross-protection, that is, a serum prepared by injection of one of the number acted on all the races of this group. A horse was then immunized to one of this group and the serum is called Serum 2. In this way it has been possible to divide the pneumococci obtained from cases of pneumonia into four groups. In Group 1 are included all the races against which Serum 1 is effective. In Group 2 are included all those against which Serum 2 is effective. Whether the races included in this group correspond with the organisms described by Neufeld as acted on by his immune *Serum Franz* is not known at present. In Group 3 are placed all the organisms of the so-called *Pneumococcus mucosus* type. These organisms have very large capsules and produce a sticky exudate in animals. In Group 4 are included all the races against which Serums 1 and 2 are not effective and which, from their other properties, do not belong in Group 3. Animals may readily be immunized to any member of this Group 4, and the serum of the immunized animal is protective against the race used for immunization. In no instance, however, has this serum been found effective against any other race of this group or against the organisms of the other groups. So far as cultural and morphologic characters are concerned, no constant group differences have been discovered between the members of Groups 1, 2 and 4. By means of the agglutination reaction, however, it has been found possible to group them in exactly the same manner as by protection experiments.

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[32] Dochez: Jour. Exper. Med., 1912, xvi, 680.

[33] Dochez, A. R., and Gillespie, L. J.: A Biologic Classification of Pneumococci by Means of Immunity Reactions, Jour. Am. Med. Assn., 1913, lxi, 727.

As previously stated, the members of Group 3 differ from the others somewhat in their morphologic and pathogenic characters. They differ further in the fact that while animals may be very highly immunized to them, the serum of such animals possesses no protective power; they induce active but no passive immunity. Studies have been undertaken by Hanes to learn on what factor this failure to produce passive immunity depends. It was found that the serum of the immunized animals not only does not protect, but also has no agglutinating power. It has been known that certain encapsulated bacilli also fail to be agglutinated by immune serum. Porges,[34, 35] however, has shown that such bacilli are agglutinated by the serum of immunized animals, provided the bacilli are previously treated so as to destroy their capsules. This method was therefore employed by Hanes[36] in studying these cocci. Six typical races of *P. mucosus* obtained from cases of pneumonia, were studied. The bacteria were treated with dilute hydrochloric acid and heated for fifty minutes at 80 C. (176 F.). The fluid was then neutralized and the bacteria so treated tested for agglutination.

TABLE II.

Agglutination of Pneumococcus Mucosus.

Organism No.	Immune Serums Nos.								Normal Rabbit Serum
	19	26	42	54	68	96	I	II	
19	+	+	+	+	+	+	–	–	–
26	+	+	+	+	+	+	–	–	–
42	+	+	+	+	+	+	–	–	–
54	+	+	+	+	+	+	–	–	–
68	+	+	+	+	+	+	–	–	–
96	+	+	+	+	+	+	–	–	–
I	–	–	–	–	–	–	+	–	–
II	–	–	–	–	–	–	–	+	–

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34. Porges: Wien. klin. Wchnschr., 1905, xviii, 691.

35. Porges and von Eisler: Centralbl. f. Bakteriol., First Abt., Orig., 1906, xlii, 660.

36. Hanes: Jour. Exper. Med., 1914, xix, 38.

Controls were made with members of Groups 1 and 2 treated in the same way. The results were definite and striking. Agglutination of all the six races of *P. mucosus* occurred promptly with all six immune serums obtained by inoculating each of a series of rabbits with one of these races. No agglutination of Pneumococcus 1 or 2 occurred with any of these serums, and *P. mucosus* was not agglutinated by either Serum 1 or 2. (See Table 2.)

These experiments show that, so far as tested, all the organisms of the *P. mucosus* type belong in one biological group differing from those of the other groups. In order to show the relation of these organisms to streptococci, the method of complement-fixation was employed. With this method there occurred a considerable amount of cross-fixation among the various races of pneumococci, including *P. mucosus,* but no cross-fixation was observed in testing the complement-fixing powers of *Streptococcus mucosus* or *S. pyogenes* serums. It therefore seems evident that *P. mucosus* is really a variety of pneumococcus, and that biologically it forms a distinct variety of this organism.

Further studies of the various members of the *P. mucosus* group, to see if any were affected by the immune serum *in vivo,* were all negative. None of the serums were able to protect mice, even against the homologous organism. These experiments and also observations of Gruber and Löhlein seem to indicate that the failure of such serums to protect is in some way related to the formation of the thick, mucoid capsules by these organisms. As soon as the

TABLE III.

Classification by Protection and Agglutination.

	Number	Per Cent.
Group 1	35	47
Group 2	13	18
Group 3 (P. mucosus)	10	13
Total typical	58	78
Group 4 (heterogeneous)	16	22
Total heterogeneous	16	22
Total number	74	

bacteria commence to grow in the body, capsules are formed which prevent the action of the immune serum. By the methods employed by Dochez and Hanes, it has been possible to study the races of pneumococci obtained from a series of cases of pneumonia. The classification by protection and agglutination experiments of sixty-two organisms so obtained gave results as shown in Table 3. In every instance in which an organism could be placed by protection in one of the groups described, the agglutination reaction has corresponded.

The races placed in Group 4 have been called heterogeneous, since each race, so far as studied, appears independent, and no grouping of the members on a biologic basis, by means of protection or agglutination, is at present possible. Table 4 shows the results of the study of agglutination with these races.

TABLE IV.

Strains of Heterogeneous Group Tested and Results of Agglutination.

Serums Pneumococcus	Pneumococcus Strain Numbers													
	1	2	34	36	37	38	52	55	60	62	71	76	82	102
1	+	0	0	0	±	0	0	0	0	0	0	0	0	0
2	0	+	0	0	0	0	0	0	0	0	0	0	0	0
36	0	0	0	+	0	0	0	0	0	0	0	0	—	0
37	0	0	0	0	+	0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0	+	0	0	0	–	0
71	0	0	0	0	0	0	0	0	0	0	+	0	–	0
76	0	0	0	0	0	0	0	0	0	0	0	+	0	0
82	0	0	0	0	0	0	±	0	0	0	0	0	+	0
Polyvalent, 34, 38, 62.	0	0	0	0	0	+	0	+	0	+	0	0	–	0

Gillespie[37] has also made a study of the various races, using the method of agglutination of bacteria by acid, as introduced by Michaelis. The results also show certain group differences in the agglutination of the various races.

Recent observations by Rosenow indicate that by certain methods it is possible completely to change the characters of the organisms of the entire streptococcus-pneumococcus group, so that one may be transformed into the other, even *S. hemolyticus* into a typical pneumococcus and vice versa. It has long been thought that the various

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37. Gillespie: Jour. Exper. Med., 1914, xix, 28.

closely related bacteria must originally have had a common source and have become differentiated by processes of adaptation. It is remarkable, however, that the changes can occur in such a short period of time as shown by Rosenow, even though they are subjected to extreme changes in environment, as has apparently been done. Some experiments performed in my laboratory several years ago by Strouse indicated that sudden mutations might appear in this group. Important as these experiments are, they do not have an immediate bearing on the pneumonia problem, except as regards the origin of the infection.

As regards the course of the disease and the possibility of specific methods of cure, the possibility of transformation of type of the organism concerned is not significant. In all the studies of organisms obtained at different times from the same case, in no instance have there been any indications of a change in type; the type first isolated has always subsequently been found. Moreover, many of these strains have now been cultivated for a long time outside the body, both in artificial mediums and in repeated passages through animals, some of them for several years, and they have in all cases retained their original characteristics. The results of the present year are not included in Table 3. This year the largest number of cases has been due to organisms of Type 2. It is possible that the prevalence of cases due to the various types varies from year to year and in different places, which may explain the variation in mortality observed in different times and places. The mortality of our limited number of cases due to organisms of different types is shown in Table 5. The most striking fact is the low mortality due to organisms of Type 4. Further observations may possibly change

TABLE V.

Mortality in Patients Infected with Varying Types of Organisms.

Infection Type	No. Patients	Patients Died	Per Cent.
1	34	8	24
2	13	8	61
3	10	6	60
4	15	1	7
Total............	72	23	32

our ideas with regard to the relative severity of cases due to organisms of the different types.

In addition to the fact that there are immunological differences in the pneumococci concerned in pneumonia, there is probably another reason why the use of immune serum has not proved efficacious in the past. The method of employment of such immune serum has been to use small doses, from 10 to 20 c.c., usually given subcutaneously. Neufeld and his assistants, by titrating immune serum against varying doses of pneumococci and making injections into mice, have concluded that in order to obtain protective power a certain proportion of serum in relation to body-weight is required. This concentration they have called the *Schwellenwert* or threshold concentration, and from experiments on mice they estimate that for man the dose of serum employed by them must be at least 77 c.c. Undoubtedly, however, this *Schwellenwert* must vary enormously under different conditions, depending on the virulence of the organism, the time the serum is given, etc. In any case their experiments indicate that the serum must be given in very much larger amounts than has hitherto been done, in order to obtain curative results. They concluded that such an antibacterial serum does not obey the law of multiple proportions. This is undoubtedly true for the conditions employed by them, namely, injections made separately in different parts of the body, and is also true for the conditions present in the therapeutic injections in man. Dochez has shown, however, that when the serum and cultures are mixed before injection, such a serum does obey the law of multiple proportions up to a certain point, but there is a maximum degree of infection against which no amount of serum, however large, is able to protect. It would therefore seem that one of the factors of the protective mechanism must be supplied by the body, and that, when the infection is very great, a sufficient number of immune bodies may be supplied by the administration of serum, but the body cannot react to a sufficient extent adequately to supply this second factor. This suggests that in order to obtain results from serum, it should be administered early, before the infection has reached too extreme a grade, beyond which no amount of serum can be effective, and also offers a possible explanation of the fact that in certain cases, such as the one which I shall mention, case 4, the serum seems to have absolutely no effect. The nature of this additional factor is not known. If, as previously stated, the serum owes much of its effect to its bacteriotropic power, the number and activity of the leukocytes may be the additional factor. That this factor may be stimulated is shown by the results of artificial immunization, whereby very much larger doses of bacteria are resisted than can be protected against by immune serum.

These experiments have indicated that for the successful employment of immune serum in pneumonia, it must be employed fairly early and in large doses, and a serum must be used which is effective against the variety of organism causing the infection. We have been able to produce a serum of very great efficiency against organisms of Type 1, and one effective against organisms of Type 2. So far it has been impossible to produce a serum effective against *P. mucosus,* and, for the reasons stated, it would only be practical to treat cases of pneumonia due to organisms of Type 4 with homologous serums. This is of less importance, however, since the cases due to these organisms are apparently of mild grade.

To employ the serum effectively in cases due to organisms of Types 1 and 2, it has been necessary to devise a method for quickly determining in each case the type of organism concerned. This has been done and the following method is employed. When a patient with pneumonia is admitted to the hospital, a culture is immediately made from the blood and also one from a portion of sputum coughed up from the lung, or, when this is not obtainable, a culture is made directly from the lung by the insertion of a needle. This procedure seems to be without danger. When there are large numbers of organisms in the sputum, a culture may be obtained most rapidly by injecting the washed sputum into the abdominal cavity of a mouse. After four or five hours the peritoneal cavity may be washed out with salt solution and the cells thrown down in the centrifuge; a suspension of the organisms is thus obtained. In whatever way the culture is obtained, the agglutination test is at once applied. If the organism fails to agglutinate with either Serum 1 or Serum 2, it is, of course, useless to undertake serum treatment. If, however, one of the serums agglutinates the organism, treatment may be commenced at once with the appropriate one.

So far it has been possible to treat only a comparatively small number of cases. Twenty-three cases have been treated with the serum. Of these, fifteen were due to organisms of Type 1 and eight to organisms of Type 2. The method of administration of the serum was the following: When admitted, the patient was given 0.5 c.c. of serum subcutaneously to discover if hypersensitiveness existed. As soon as the type of organism was determined, from 50 to 100 c.c. of the serum, diluted one-half with salt solution, were injected intravenously. The condition of the patient served as a guide in the later treatment. Usually the serum was not administered oftener than every twelve hours. The patients treated received totals of from 190 to 460 c.c. of serum, except one, who received a total of 700 c.c. of serum. The patients treated were all

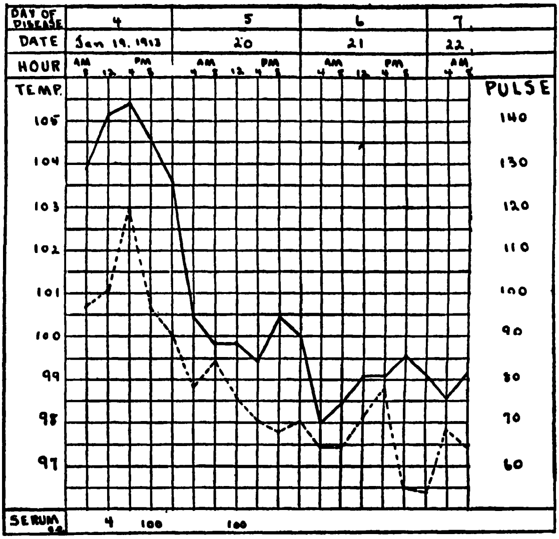

Fig. 1.—Case 1, E. S., No. 988, aged 20 years, admitted January 18, 1913, on the third day of the disease. Physical signs showed involvement of the lower left lobe and also signs of beginning involvement in the lower right lobe. Blood culture was negative. Pneumococci obtained from sputum were of Type 1. The patient was quite ill on admission, respirations were labored and there was cyanosis. His condition was markedly improved on the day following the first administration of the serum, as shown by the temperature and pulse curves. Recovery was uneventful except that slight signs in the lungs persisted for several weeks.

seriously ill. They were treated in series. Every case infected with a pneumococcus of Type 1 or Type 2 was treated. Of the fifteen cases due to Pneumococcus 1, all of the patients recovered but one, and of the eight cases due to Type 2, two patients died. One of these patients objected to the treatment and would not allow its continuation, so it was not thoroughly carried out. When we consider that the mortality among the untreated patients infected with Types 1 and 2 is very high (Table 5), the result is certainly not discouraging. It must also be remembered that so far most of our cases have been admitted late in the disease. Treatment was commenced on the third day in six cases, on the fourth day in five cases, the fifth day in six cases and the sixth day in six cases. If treatment can be commenced early, it is probable that the results will be even better than they now are. It is to be hoped that during this winter a large number of cases may be treated early in the disease. Effective treatment in the cases due to Types 1 and 2 should cut down the total mortality due to pneumonia very materially, as it has already done in our hands. I prefer at present, however, not to lay the

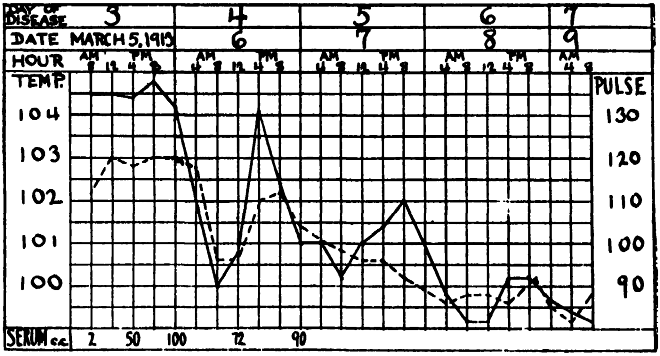

Fig. 2.—Case 2, B. G., No. 1175, aged 36 years, admitted March 4, 1913, on the second day of the disease. There was slight involvement at the base of the right lobe. Blood culture was positive. Agglutination test showed Type 1 organism. Treated with serum on day following admission. The signs of involvement in the right lower lobe became more distinct, but there was no apparent extension of the involvement beyond this lobe during the course of the disease. Following injection of the serum the patient's general condition improved. The patient complained of some urticaria and joint pains beginning on the twelfth day.

main stress on the mortality statistics, since these are not large enough to be conclusive, but to refer to other criteria which we possess as to the efficacy of the serum.

Let us first consider the effect on the clinical course of the disease. Following practically all the injections, a reaction has occurred. The temperature usually rises and then falls, but does not necessarily remain low. In two instances the rise of temperature has been marked. In the other cases the rise of temperature following the injection was only a degree or so. In all the cases except the fatal ones, the serum has apparently had an ultimate favorable effect in lowering the temperature and shortening the course of the disease, though, of course, this is a very difficult matter of which to be absolutely sure. In no case was one injection of the serum sufficient to bring on a crisis.

Figures 1, 2, 3 and 4 show the effect of treatment on the temperature curves in certain of the cases. It is manifest that wrong

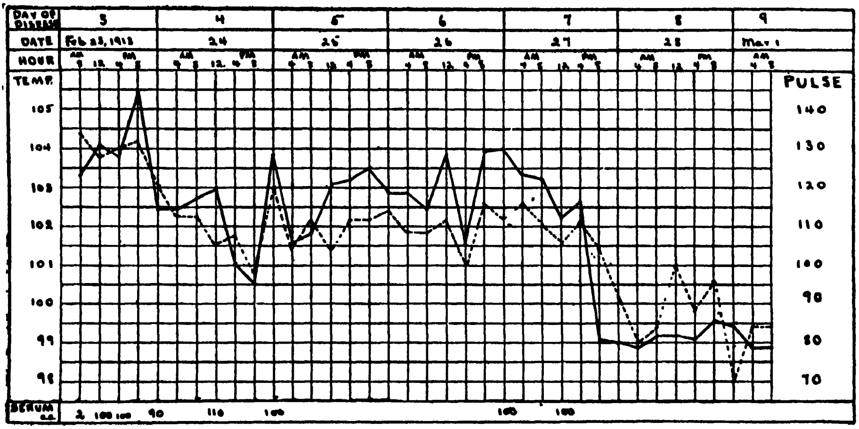

Fig. 3.—Case 3, M. L., No. 1091, aged 30 years, admitted February 22, 1913, on the second day of the disease. Blood culture showed a growth of pneumococcus, Type 1. Physical signs indicated involvement of the right lower lobe with slight involvement of the left lower lobe. The patient's condition improved following the treatment and the signs in the lungs became less well marked. On the sixth day, however, the patient's condition was again worse, the temperature was higher and the pulse more rapid, so that treatment was commenced again, following which the patient's condition markedly improved and he made a rapid recovery except for symptoms due probably to serum sickness on the twentieth to the twenty-fifth days of the disease.

impressions may be produced by the exhibition of temperature curves unless all the curves of a series are given. To avoid this difficulty, so far as possible, however, a curve from each group of cases is shown. Figure 1 represents the curve of a case in which the serum apparently had a marked effect, the temperature falling promptly and in a striking manner. Figure 2 indicates a temperature curve in a case in which the temperature fell following the administration of serum, but several doses were necessary before the temperature remained low. Figure 3 shows the temperature curve in a case in which there were apparent effects of the administration of serum, but after the administration of serum was discontinued, the temperature curve rose and only fell after further large

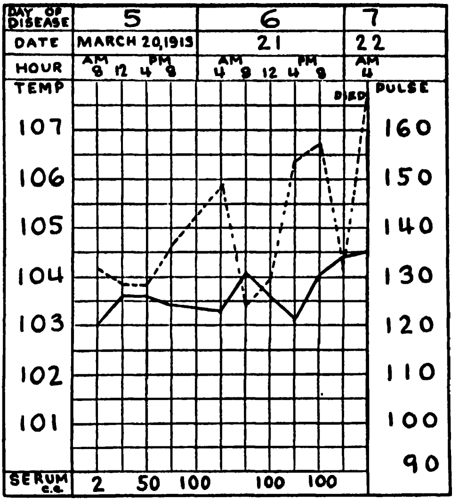

Fig. 4.—Case 4, F. W., No. 1090, aged 36 years, admitted March 19, 1913, on the fourth day of the disease. The patient was extremely ill on admission. There was involvement of the right upper and lower lobes. Blood culture and culture from the lung showed the presence of pneumococcus, Type 1. There was some irregularity of the heart. Following treatment on the fifth day, the patient seemed better. The blood culture was negative. The pulse continued rapid and showed an extrasystolic irregularity, however, and the patient showed a very marked cyanosis. In spite of the active serum treatment, the patient died on the seventh day.

doses of serum were administered. In Figure 4 is given a temperature curve in which the serum had apparently no effect and the patient died on the seventh day.

All the patients seemed to feel better following the injection of the serum, and in a number of cases the apparent lessening in the degree of intoxication was very manifest. When the treatment was commenced early, no extension of the involvement of the lung occurred. On the other hand, there was no special tendency in the treated cases for the lung lesion to resolve rapidly. If anything, there seemed to be a tendency for resolution to be delayed in these cases. This has been noted by others in certain cases treated by serum.

More important than the foregoing criteria, however, as indicating an effect of the serum, are the following observations, since they have depended solely on objective procedures: First to be mentioned is the effect of the serum on the organisms in the blood. In ten cases pneumococci were isolated from the blood before the treatment was commenced. In all cases blood-cultures were made before each treatment; and in all of these ten cases after one treatment and before the second (or within from eight to twelve hours) the blood had become sterile. The conclusion seems justified, therefore, that one large dose of active serum given intravenously is sufficient to sterilize the blood. It also seems certain that if the organisms are not present in the blood, the administration of the serum will prevent their invasion. Second, in a previous study of the protective substances in the blood-serum of patients with pneumonia, it has been shown that, as a rule, the appearance of protective substances in the blood, when demonstrable, coincides rather sharply with the period of critical fall in temperature and the disappearance of symptoms. Before the crisis they are not present in the blood in any measurable degree.

A similar study has been made by Dochez of the protective substances in the serum in a number of the cases of pneumonia treated with serum. In all the cases studied, it has been possible to demonstrate the appearance of such substances in considerable amounts in the serum very shortly after the administration of one dose of the immune serum, even when this serum has been administered early in the disease, at a period when such protective substances are otherwise never present (Table 6). These substances persist, and in case they play a part in the mechanism of recovery, as was concluded

TABLE VI.

Protective Power of Serum B. G.; Treatment Commenced on the Third Day.

Quantity of Culture in c.c.	Virulence; No Serum	Quantity of Serum in c.c.	Serum Obtained			
			Third Day, Before Treatment	Third Day, 6½ Hours After Treatment	Fourth Day, After Treatment	Ninth Day, Five Days After Last Treatment
0.001	—	0.2	16†	*	*	† Days
0.0001	—	0.2	20†	† 5 Days	*	*
0.00001	24†	0.2	24†	*	*	*
0.000001	28†	0.2	24†	*	*	*

* Animal protected as shown by survival.

† Time in hours before death of animal injected.

from the previous study, it is evident that their appearance indicates a favorable action of the immune serum.

The results obtained, therefore, from the clinical and laboratory study of this series of cases of pneumonia treated by the injection of large amounts of appropriate serum, seem to indicate that a method has been devised for the successful specific treatment of at least a portion of the cases of acute lobar pneumonia. Studies on the treatment of pneumonia by the intravenous injection of the Neufeld-Händel immune serum have been made by Beltz,[38] Weitz[39] and Geronne.[40] In none of these series, however, were studies made of the type of organisms concerned in the infection of the cases treated, and in all of the cases the amount of serum administered was too small, judging from our own experience, to be of value.

The mode of action of the immune serums is still somewhat obscure. It is quite evident that there is an antibacterial action, inasmuch as the bacterial invasion of the blood is prevented. The action on the local lesion, however, is less evident. It is probable that here the organisms can less readily be reached by the serum, though apparently in most cases the growth of the bacteria in the margins of the lesion has been inhibited, as shown by prevention of spread of the process. In addition to the antibacterial action, the clinical cases show a definite change as regards intoxication. It is possible, of course, that this is entirely associated with the destruc-

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38. Beltz: Deutsch. med. Wchnschr., 1912, xxxviii, 14.
39. Weitz: Med. Klinik, 1912, viii, 1072.
40. Geronne: Berl. klin. Wchnschr., 1912, xlix, 1699.

tion of the organisms. Certain experimental work, however, has indicated that the serum may possess some antitoxic effect.

When the immune horse-serum is added to the toxin obtained by dissolving pneumococci in bile, it is found that such a serum has a well-marked effect in inhibiting the hemolysis of sheep corpuscles by this toxin. When it is added to the toxin in doses of 1 c.c. of serum to 4 c.c. of toxin and placed at 37 C. (98.6 F.) for one-half hour, the effect of the toxin when injected into guinea-pigs is diminished or entirely prevented. These effects of the immune horse-serum are much less specific, however, than are the protective or antibacterial effects, since the Serum 1 acts on both Toxins 1 and 2, though most markedly on Toxin 1. Serum 2 also shows a similar diminution in specificity in antitoxic action as compared with antibacterial action. These experiments offer some evidence that part of the effect of the immune horse-serum is antitoxic, admitting, of course, that the toxic substances obtained from the bacterial bodies are responsible for the intoxication.

An effort has been made to obtain a pure antitoxic serum by the injection of the toxin alone into animals. Rabbits have been immunized by the repeated injection of this toxin and a sheep also has been highly immunized. The sheep-serum and also the immune rabbit-serums show antitoxic power, as indicated by anti-hemolytic action and also by neutralizing effect on the toxin, as tested by injection into guinea-pigs. The effects, however, are less marked than those of the antibacterial horse-serum. These antitoxic serums are also protective against the living organisms, as shown by tests on mice. The protection, however, while fairly high, is less well marked than that of the horse-serums. The protection is not so specific as that of the horse-serum, since the serum produced by the injection of toxin prepared from an organism of Type 1 is not only protective against this organism, but also, though to a less extent, is protective against organisms of Type 2.

The interpretation of these experiments is attended with much difficulty. It is possible that these antitoxic serums may show protective power only because living organisms were introduced, since in the preparation of the toxin one cannot be positive that all organisms have been destroyed. These antitoxic serums, however, possess no power to cause agglutination, and this fact, together with their lessened specificity, suggests that we are dealing with serums which owe their power to other properties than those of the antibacterial serums. The experiments are of importance, moreover, since they indicate that immunity may be obtained against the substances contained in the bile extracts, and since the essential criterion of a toxin in the Ehrlich sense is that immunity may be obtained to it. Much more work will have to be done before such antitoxic serums should be employed therapeutically.

It is probable that in the future it will be possible to obtain the same therapeutic effects by the injection of much smaller amounts of serum than are now employed. Work now being carried on by Avery shows that the immune substances are all contained in the globulin fraction of the serum, and methods are now being devised for the concentration of the serum, so as to avoid the injection of a very large part of the serum protein which contains no immune substances. In this way it will probably be possible to avoid serum sickness, which has occurred in a number of our patients in from ten to twelve days after the administration of the large amounts of horse-serum. This serum-sickness, while causing some discomfort to the patients, is not of any serious import, so far as we know.

It may be possible later to produce polyvalent serums that are efficacious. At present, however, and until the value of the special serum in the cases due to organisms of Groups 1 and 2 is unquestionably determined, it does not seem to be advisable to make such attempts. The objection is frequently raised that this method of treatment is very complicated. One may reply to this that so is the treatment of appendicitis.

At the present time I can do no more than mention the efforts along other lines that have been made to produce curative results by specific measures. Most important studies were made by the late Professor Hiss in the treatment of bacterial inflictions by means of leukocytic extracts. So far as concerns pneumonia, the results of experiments on animals are not very convincing, but the brief clinical report of cases of pneumonia treated, as stated in the article published since his death, seems extremely favorable and promising. It is to be hoped that study along this line will be continued.

Lamar has devised a method for the local treatment of pneumococcus infections. He has shown that immune serum has a much greater effect on pneumococci treated with sodium oleate solutions than on cocci simply washed in salt solution. This action of the soap, however, is inhibited if the serum be added first or mixed with the soap solution before treating the bacteria. The inhibiting action of serum, however, may be prevented by the addition of small amounts of boric acid, as Liebermann and von Fennyvessy have shown. By combining the soap, serum and boric acid in proper concentrations, Lamar has found a mixture that is much more efficacious in the local treatment of experimental pneumococcus infections than is serum alone. The treatment of local infections, as meningitis, with such a mixture, using serum effective against the race of organisms concerned, should be tried in all suitable cases. It is doubtful, however, whether such a mixture can be employed intravenously.

A final possible method which may be rendered practical in the treatment of pneumonia is along the lines of chemotherapy, as laid down by Ehrlich. It has been generally held that such a method of treatment may be of value in protozoan infections, but not in diseases due to bacteria. Morgenroth[41] and his co-workers, however, have shown that a derivative of quinin—ethylhydrocuprein—has a specific action on pneumococcus infections in mice, and Wright has shown that this drug is bactericidal for pneumococci in the test-tube. The drug has been employed clinically, but cases of amblyopia developing have indicated that the toxic dose in man too closely approaches the curative dose to permit the safe administration of the drug. It is possible, however, that with further study, its toxic properties may be reduced without lessening its curative effect.

CONCLUSIONS.

Much obscurity still exists concerning the mode of natural infection in pneumonia, though by animal experimentation many facts in regard to it have been discovered.

The symptoms in pneumonia are probably due to toxic substances derived from the bacterial cells.

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[41] Morgenroth: Berl. klin. Wchnschr., 1911, No. 44.

The outcome is dependent on the virulence of the organisms concerned and on the ability of the body, first to limit the local infection, and second, to prevent the invasion of the blood by the organisms, as on the latter the outcome of the disease mainly depends.

Leukocytes probably play a part in the resistance, certainly as regards the local spread and probably also to some extent as regards the general infection.

The most important part in prevention of the general infection is probably played by immune substances contained in the serum. Such substances are present in the serum of immunized animals.

Pneumococci differ in regard to their immunological reactions and on these they may be divided into several groups.

In order to use immune serum effectively in treatment, as in prevention, it is necessary to employ the serum effective against the group of organisms to which the special organism causing the infection belongs.

Immune serums effective against two of the most important groups have been produced. This treatment has been carried out in a limited number of patients with promising results.

It is probable that the methods of application of such serums will be improved, and it is possible that the method may be combined with other measures directed toward other factors which are important for the outcome. In any case, facts regarding the nature of the disease are being disclosed, and the outlook, at least for lessening the ravages of this dreadful disease, is encouraging.

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STUDIES CONCERNING DIABETES.*

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The work, of which this paper will give a synopsis, is the continuation of an experimental study of diabetes described in detail elsewhere.[1] Certain of the results of the previous investigation, bearing on the present topic, may be summarized under the following four headings:

1. Production of Diabetes: Removal of portions of the dog's pancreas produces a lowering of the sugar tolerance. Removal of nine-tenths of the gland results in severe diabetes. When the remnant is larger (for instance, one-eighth), milder types of diabetes result. The course is chronic, extending over months, and the end fatal. Such animals furnish a closer imitation of human diabetes than is produced by total pancreatectomy.

2. Pathologic Anatomy of Diabetes: In the foregoing procedure, duct communication between the pancreatic remnant and the duodenum is preserved. Therefore the acinar tissue does not atrophy, and in some cases it may hypertrophy. The islands of Langerhans show typical progressive alterations, which may be summarized as vacuolation of cytoplasm and loss of granulation, pyknosis of nuclei, loss of cells, and finally disappearance of islands. By suitable controls the changes were shown to be specific to diabetes. Homans[2] has made a careful histologic study of such changes in the islands of diabetic cats by the aid of the admirable methods of Bensley.

3. Pathologic Physiology of Diabetes: An idea has long existed

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* Read before the Section on Pathology and Physiology at the Sixty-Fifth Annual Session of the American Medical Association, Atlantic City, N. J., June, 1914.

[1] Allen, Frederick M.: Studies Concerning Glycosuria and Diabetes, Harvard University Press, Cambridge, Mass., 1913.

[2] Homans, John: Jour. Med. Research, 1914, xxx, 49.

that the disturbance of carbohydrate metabolism in diabetes might perhaps be explained by the assumption that sugar exists in a combined form in the normal body, but that this combination is lacking in diabetes. In the diuretic behavior of dextrose in diabetic and non-diabetic animals, I found contrasts which were interpreted as evidence in favor of this conception, which was therefore put forward as a hypothesis to explain the various phenomena of diabetes. The combining substance was designated by the figurative title of "amboceptor," to indicate its function as a bond between tissue and sugar. The substance which thus combines with dextrose, or some link in the combination, is supposed to be furnished by the islands of Langerhans. For details concerning the hypothesis and the evidence, reference must be made to the original publication.

4. Prevention or Checking of Diabetes: Under certain conditions the presence or absence of experimental diabetes may be determined by the patency of the pancreatic duct. When the pancreatic remnant is sufficiently small, diabetes results, even if this remnant is cut off from duct communication. But, with a slightly greater size of pancreas-remnant, it is possible under favorable conditions in certain cases to show that diabetes occurs if the duct is patent, but remains absent if the duct is ligated. Particularly in one instance it was shown that a diabetes already begun stopped when the duct was ligated. The period during which diabetes can thus be prevented is necessarily limited, since ligation of the duct produces in the dog gradual atrophy and degeneration of all the elements of the pancreas, and this gives rise after weeks or months to the Sandmeyer type of diabetes.

PRESENT INVESTIGATION.

The present research has advanced along the same lines as the preceding one, and has kept in view the same clinical purpose.

I. PRODUCTION OF EXPERIMENTAL DIABETES.

A. By Operation.

Of several species of animals tried, the dog and cat are the most convenient and useful. Rapid death from cachexia, which I previously described as an occasional occurrence in dogs, is more frequent in cats. In them diabetes is intense and life short after removal of nine-tenths of the pancreas; but by removal of fractions such as three-fourths, four-fifths and five-sixths of the pancreas, one obtains mild or severe forms of diabetes as desired, and such cats live for weeks or months. In cats, more readily than in dogs, can thus be produced mild, transitory, intermittent forms of glycosuria, doing the animal little or no harm, and determined largely by diet (as milk, or sometimes even the feeding of liver instead of ordinary meat). There is a decided tendency toward recovery, though aggravation can often be produced by continuance of improper diet. This form of diabetes may be compared to those mild, intermittent, relatively benign human cases which some persons have supposed to be not of pancreatic origin.

With respect to dogs, the figures given above concerning size of pancreas-remnants hold good for medium-sized animals, such as were convenient for the purposes of the previous research. Present findings indicate that the size of the pancreas in proportion to body-weight in large and small dogs may vary considerably, and that the size of the pancreas-remnant which permits diabetes may vary correspondingly, though a longer series of observations must be completed before making more precise statements. In cats variations of this character are absent or less marked.

B. By Diet.

The papers of Thiroloix and Jacob, previously reviewed, report success in a line of experiment in which earlier workers failed. They state that some dogs after partial pancreatectomy are free from glycosuria on meat diet, but show glycosuria when carbohydrate is fed; and that continuance of carbohydrate diet finally brings the animals into a state of severe diabetes in which they excrete sugar even on meat diet. The reports of the French authors are brief, and contain no mention of controls or proof that such dogs might not ultimately develop severe diabetes, even without carbohydrate feeding. These may perhaps be the reasons why this discovery has received practically no notice in the literature. Independently of these writers I had made similar observations, and believing them to be of importance, I have repeated and extended the work. Based on the experiments so far conducted, it is possible to make the following statements:

1. After removal of sufficiently large fractions of the pancreas, as above described, dogs develop a severe diabetes, in which they show heavy glycosuria on meat diet and also during considerable periods of fasting. The condition progresses steadily downward to a fatal end.

2. When the remnant of pancreas left *in situ* is slightly larger, a condition may be produced in which the fate depends on the diet. On meat feeding such a dog is free from glycosuria and remains so for months, eating his fill every day and maintaining full health and nutrition, with no sign of downward progress; but subcutaneous tests show that the dextrose tolerance is very low, and bread feeding readily produces glycosuria. A return to meat diet stops the glycosuria; but if the bread diet and accompanying glycosuria are maintained for too long a time, the glycosuria then continues, even on meat feeding. The diabetes thus produced is not inferior in severity to that resulting from simple removal of larger fractions of pancreatic tissue, and the downward course and fatal termination are similar.

3. When the pancreas-remnant is still larger, glycosuria is absent on meat diet, and on bread diet may be absent or transitory. Such animals may remain in excellent condition indefinitely on bread diet, free from glycosuria or any downward tendency; but if sufficient sugar is added to the diet, glycosuria can be produced and maintained. After a period of such glycosuria, the animal reaches a condition in which it is glycosuric on bread diet. By prolonging the glycosuria on bread diet, the dog finally reaches the condition of severe diabetes, with glycosuria on meat diet, and continuous downward progress. For such sugar feeding I have ordinarily used commercial glucose. The experiments succeed best in greedy dogs and those naturally fond of sugar. A decided aversion to sugar on the part of the dog may spoil such an attempt through failure of appetite.

4. When the pancreas-remnant is still larger, sugar feeding may produce transitory glycosuria, but it cannot be made to continue.

The sugar tolerance is lower than in normal dogs, but nevertheless the doses of sugar necessary to produce glycosuria are higher than can be tolerated as a daily routine by the gastro-intestinal canal. Persistence in the attempt to maintain glycosuria causes diarrhea and illness. The dog refuses to eat, sugar given by stomach-tube is vomited, and true diabetes remains absent. Similar results can be obtained with cats by feeding carbohydrate in the form of milk.

The changes previously described as occurring in the islands of Langerhans occur whether the diabetes follows directly on the operation alone, or has been induced in the prepared animal by means of modification of diet. The best of the control animals are still alive; but the control tissues thus far examined indicate that in animals predisposed by operation, when diabetes is prevented by carbohydrate-free diet, the signs of exhaustion and degeneration in the islands of Langerhans are absent. If further study shows that this is the case, it will afford additional evidence that these changes have a direct relation to the diabetes and that they are a result of functional overstrain.

While various factors are undoubtedly concerned in the production of human diabetes, clinical observations indicate that diet is an important one. It is generally recognized that there is a higher incidence of diabetes among those races or classes of people who use an excess of carbohydrate. Luxurious living and sedentary life are thought to predispose to this disorder. The experimental observations on animals suggest an explanation of this relationship between diet and diabetes in the human subject. If individuals differ in the strength of the pancreatic function as in other functions, in some this may be so weak that diabetes comes on in early life irrespective of the diet. In others this function may be only a little stronger, so that diabetes may be delayed until later in life or even to the period of senility, when there occurs an impairment of various functions. Others may be more or less below the average in pancreatic functional power, but under ordinary circumstances this is sufficient to prevent the occurrence of diabetes. In these persons, however, who might otherwise go through life with no sign of diabetes, an excess of starch in the diet may serve as an exciting cause of diabetes, and the degree of such excess may help to determine the earlier or later onset and the milder or severer type of the disorder. In other persons of this sort, as in the corresponding type of dogs, the pancreatic function is able to deal safely with as much starch as can be digested, but a sufficient excess of sugar is an effectual cause of diabetes.

It is impossible to produce diabetes in the normal dog by an excess of carbohydrate feeding, since if too much sweet or starchy food is taken, indigestion results and automatically stops the ingestion. But in dogs after operation and in predisposed human patients, pancreatic weakness may reverse the normal relation, so that the organism can digest and absorb more carbohydrate than it can combine and assimilate; and in this condition the production of diabetes by improper diet is possible. The question of diabetes may then be regarded as a balance between the digestive and assimilative functions. Moreover, the body is equipped with no natural safeguards against this unnatural condition. There is no reflex or other mechanism which points plainly to the cause of the trouble and compels its cessation. On the contrary, the impaired nutrition due to failure of assimilation affects the organism as if it were due to deprivation of food. The response commonly shows itself in the harmful form of increased appetite. The taking of excessive food injures the assimilation still further, and thus a vicious circle results.

II. PREVENTION OR CHECKING OF EXPERIMENTAL DIABETES.

The therapeutic experiments in animals pertain to two groups of cases, those in which diabetes is produced by simple operation, and those in which diabetes is produced by diet in predisposed animals.

1. *Animals Made Diabetic by Operation.*—In animals from which an excessive amount of pancreatic tissue is removed, a diabetes may be obtained which is so severe that fasting will not produce sugar-freedom. But when the pancreas remnant is of suitable size, for example, one-tenth, though the resulting diabetes is permanent on meat diet and will end fatally if allowed to persist, nevertheless a few days of fasting at the outset will produce sugar-freedom. If the diabetes is allowed to continue longer, a much longer period of fasting may be necessary for sugar-freedom, or it may be impossible to

obtain. If, after obtaining sugar-freedom, feeding of protein and fat (with occasional bones) is begun very cautiously, in quantity only enough to maintain the animal in its thin condition, such dogs remain free from diabetes. The longest experiment to date is that of a dog which, possessing less than one-tenth of the pancreas, has been kept free from diabetes for six months, and there is at present no indication that the condition cannot be continued indefinitely. If an attempt is made to increase the weight of such an animal, glycosuria soon appears and must be checked by renewed fasting. Such dogs, though very thin, are vigorous and lively. They contrast sharply with dogs which, after similar operations, are allowed to remain diabetic on full meat diet. Though the latter animals at first appear much better nourished, they finally emaciate in spite of the most enormous eating; and when they have become as thin as the dogs above described, weakness and cachexia are very evident, and the progress continues downward to death.

2. *Animals Made Diabetic by Diet.*—When the animal possesses perhaps an eighth or a sixth of the pancreas, and is not diabetic after operation but is then made diabetic by excess of carbohydrate, this diabetes at first can be stopped by a simple change to meat diet. After a longer duration, the diabetes no longer stops on carbohydrate-free diet; but after a period of fasting, the time depending on the severity of the diabetes, the urine becomes sugar-free. With still greater duration or severity of the diabetes, sugar-freedom is no longer obtainable by fasting. When such animals are made sugar-free, they may be kept so by the same procedure as described for those of the former group. When the diabetes is thus checked fairly early, it is evident that the prognosis is much more favorable than in animals of the previous group, for in the present animals the removal of pancreatic tissue was considerably less, and the cause of the diabetes is partly a functional change. Accordingly, it is found that the diet can be gradually increased, and in favorable cases the animals be brought to a higher level of weight and nutrition than is ever possible with dogs of the preceding type. Up to the present, however, the experience has been that these dogs are never able to return quite to the condition which they enjoyed before the diabetes, and which is permanently maintained by similar dogs in which dia-

betes is prevented by suitably chosen diet from the outset. As above mentioned, dogs so treated can be kept indefinitely at full weight and well-being when placed after operation on a diet which keeps them free from glycosuria. But when diabetes gravis has been produced and allowed to continue long enough to demonstrate its reality, while it has been possible by the foregoing method to stop the diabetes and bring a dog back to within a kilogram of its normal weight, every attempt to produce a further gain in weight has brought a return of glycosuria, which must be checked by fasting. Such dogs, however, are so near to normal that, if they are mixed up in a yard with ordinary dogs, a stranger might be unable to tell one from the other.

The microscopic examinations of the pancreas remnants of such animals as have yet come to necropsy from both of these groups show that in both alike the island cells are well endowed with cytoplasm and granules, and degenerative processes are not seen; but there is a decided impression that the islands are inferior in both size and number to those of normal animals. If this impression proves valid in further experiments, a correlation readily suggests itself between the observed anatomic change and the diminished pancreatic function described in the preceding paragraph.

In my previous report I have shown that by removal of suitable fractions of the pancreas, animals may be brought so close to the verge of diabetes that the removal of less than a gram of additional pancreatic tissue suffices to bring on diabetes. According to the "amboceptor" hypothesis, the pancreas furnishes some definite substance or substances used by the cells of the body for the metabolism of carbohydrates and perhaps also of other foods, the "amboceptor" being used up in metabolism and bearing some quantitative relationship to the metabolism. Extending this theory to the foregoing experiment, one may say that the fragment of pancreas which barely prevents diabetes is the smallest fragment which can supply the minimum quantity of "amboceptor" necessary for the animal's metabolism. When such a pancreatic fragment is even slightly reduced, the supply becomes slightly deficient in quantity, and diabetes accordingly begins and runs the usual downward course.

Present observations prove that the reverse of this experiment is likewise possible, that is, when an animal and its food consumption are suitably reduced, a pancreatic remnant otherwise inadequate becomes adequate; diabetes under these conditions may be prevented or be checked after it has appeared. Prevention or cessation of diabetes previously observed in consequence of ligature of the pancreatic duct may be explained by the impaired food absorption and chronic malnutrition of the animal. Similarly in dogs which develop distemper after the pancreatic operation, and which therefore refuse food and emaciate rapidly, diabetes may fail to appear or may stop after it has appeared. Interpreting these experiments on the basis of the "amboceptor" hypothesis, one may say that an otherwise insufficient amount of "amboceptor" may become sufficient when the metabolism of the body is artificially diminished.

III. CLINICAL TREATMENT OF DIABETES.

In the clinical literature of diabetes there are authentic reports of certain cases in which even severe diabetes has cleared up spontaneously and completely on the onset of cirrhosis of the liver, cancer, tuberculosis or some other wasting condition. Other cases have notably improved. Probably the most severe and certainly the best studied of such examples was presented by one of Joslin's patients,[3] in whom a long-standing and dangerous acidosis disappeared entirely and a negative carbohydrate balance became positive, following the onset of tuberculosis. When these cases are considered in the light of the experimental study of animals, the possibility is suggested that we have an indication for a rational method of dealing with diabetes, or impaired pancreatic function associated with overstrain.

Notice should be taken of the advantage possessed by most diabetic human patients over diabetic dogs. Even the most hopeful of the above-described dogs has only a small fraction of the pancreas. Most of the organ is hopelessly gone, and the presence or absence of diabetes is determined by functional influences acting on the little remnant. Furthermore, even when the diabetes is inaugurated by functional means, the organic degenerative changes quickly ensue in the islands of Langerhans. Under these conditions it is surprising that results are obtainable by treatment at all. On the contrary, in typical human diabetes the entire pancreas is present, and there are indications that functional disturbance is an important factor.

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[3] Benedict, Francis G. and Joslin, Elliott P.: A Study of Metabolism in Severe Diabetes, Carnegie Institution of Washington, 1912, Case R, p. 55.

It has been thought justifiable, therefore, to undertake the treatment of a limited number of patients by a method based on the principles derived from the experimental work as indicated in this communication. The number of patients so far treated is limited, but the results obtained indicate that the same method employed in rendering the diabetic dog free of glycosuria and prolonging its life is efficacious in eliminating glycosuria and acidosis in the human patient. To what extent life may be prolonged by this method only a large statistical study will show. The observations so far indicate that the method is not harmful, and when carried out carefully seems definitely beneficial.

The method of treatment is in brief as follows: If the patient is moderately emaciated, with a negative carbohydrate balance and acidosis, he is put to bed and receives no food whatever. If coma seems imminent the usual emergency treatment with purging, stimulants, alkalies and large amounts of water should, of course, be carried out. In addition to fasting, alcohol is important in the treatment at this stage. From 50 to 250 c.c. of whisky or brandy may be given in each twenty-four hours in small doses, from 10 to 20 c.c. every one to three hours during the twenty-four. As soon as the glycosuria stops and the acidosis diminishes, which even in severe cases may be within forty-eight to ninety-six hours, the amount of alcohol and alkali may be reduced. Fasting and moderate dosage of alcohol are continued for from twenty-four to forty-eight hours longer, however, depending on the patient's strength. The alkali is now stopped, and feeding with starch is commenced in order to clear up the last traces of ketonuria. The kind of starch is of minor importance. Green vegetables are useful because their carbohydrate and food value is so low that they can be given in considerable bulk, and this bulk is agreeable to the patient for relieving his feeling of emptiness. Neither fat nor protein is added. For the first day, the food is chosen to represent a carbohydrate content of from 10 to 40 gm. This is divided into four to ten equal portions and fed at equal intervals during the day. If glycosuria remains absent, the ration for the next day is doubled, to represent 20 to 80 gm. of carbohydrate, similarly divided into numerous small portions. On the next day it is sometimes possible to increase the ration to 100 gm. of carbohydrate, without glycosuria. About this time, especially if glycosuria has appeared, another fast-day is interposed, from 50 to 200 c.c. of whisky being given. Present experience indicates that even in severe cases ketonuria may by this method be made to disappear entirely. Several repetitions of the foregoing routine may be necessary for this purpose. All food contains danger, tending toward either glycosuria or ketonuria. The carbohydrate of the diet is seldom reduced below 50 gm., and is preferably kept higher. If carbohydrate must be kept low, the total diet is kept low. The diet is so chosen that glycosuria, not ketonuria, is the signal of overstrain. Fasting-alcohol days are given not merely whenever this signal appears, but also at close enough intervals to prevent it from appearing, even every two or three days if necessary. If there has been no glycosuria, a slight addition to the diet is made after each fast-day. Each day's diet is calculated exactly, and the nitrogen-balance is watched. It is thought that no matter how low the assimilative power, the attempt to feed in excess of this power is harmful, and it is possible that by rest the assimilative function may gradually become stronger. With improvement in the patient's condition, the carbohydrate in the diet is further increased. Increase in weight, however, is not attempted at this time. From our present point of view, contrary to the generally held opinions, the attempt to increase weight should be the last rather than the first step in treatment. It is attempted to keep the metabolism at the lowest safe level until the patient is taking from 100 to 150 gm. of carbohydrate (mostly as green vegetables) daily, with fast-days interposed often enough to prevent any trace of glycosuria from appearing. Then protein is cautiously added, always being kept rather low; and in favorable cases the weight and well-being may finally improve under gradual additions of fat.

The radical procedure here described is that used for the most severe cases. In milder cases the treatment may be correspondingly milder. Primary loss of weight is intentional. The purpose of the treatment is not to confer temporary comfort or appearance of well-being, though various symptoms, including polyphagia and asthenia, may actually be relieved. When there is extreme cachexia and emaciation, the difficulty is greatest. It may then be necessary to juggle very carefully the three factors of glycosuria, acidosis and nutrition.

A report giving the results of this form of treatment in a series of cases will be published later. It is felt that the conception underlying this method of treatment, based on experimental observations, is new, though certain details have long been recognized as of importance. According to this method, alkali treatment is not employed, unless for a brief period at the outset, while severe acidosis is being combated. The alkali treatment has been called the most brilliant discovery in the modern study of diabetic therapy. It is indeed a valuable means for facilitating the excretion of acetone bodies. But under an efficient treatment of diabetes, acetone bodies should not be excreted. They should be burned.

Though it seems possible thus to check all active symptoms, with apparent benefit, even in very severe cases, yet it is felt that the ultimate outlook for these patients is far less favorable than it would be if they could be treated earlier. The best therapeutic hope is believed to lie in the application of this principle of treatment at the earliest possible stage in diabetes.

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PNEUMOCOCCUS HEMOTOXIN.*

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It has been generally held that pneumococci or their products do not possess the power of producing lysis of red blood cells. On the other hand, it has long been known that the culture filtrates of certain bacteria, such as staphylococcus, certain races of streptococcus, and, above all, *Bacillus tetani,* possess this property to a marked degree. The properties of the hemolytic toxin produced by the last named organisms have been thoroughly studied by Madsen and others, and through this study many of the facts concerning hemolytic toxins have been discovered. Since such hemolytic toxins may be filtered, and since they may act as antigens, they may be considered true toxins in the Ehrlich sense. Old cultures of other bacteria, such as *Bacillus pyocyaneus* and *Bacillus anthracis,* may also be hemolytic, but the production of true hemolytic toxins by these organisms is considered doubtful. Indeed, very old cultures of practically all bacteria may produce hemolysis, but it is possible that this lytic effect, in certain of the cases at least, is due directly to changes in reaction of the old culture medium.

The property of certain races of streptococci of producing hemolysis has been considered by Schottmüller and others to be of great value in differentiating between the different varieties, and also between pneumococcus and the virulent streptococci, the so called *Streptococcus hemolyticus.* The usual method of determining whether bacteria possess this property is to grow them on agar plates containing blood, when in the case of hemolytic bacteria, such as *Streptococcus hemolyticus,* the colonies become surrounded by a transparent zone contrasting with the opacity of the rest of the medium, while in the case of non-hemolytic bacteria, such as pneumococcus, no such clear zones are seen.

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While the power to produce hemolysis in culture medium is not possessed by pneumococci, or, if so, to a very slight degree, observations which we have made indicate that the bodies of pneumococci contain a substance or substances, which when set free are actively hemolytic, and that the serum of animals immunized to the bacteria or to the bacterial substance has increased power of neutralizing this lytic poison. These lytic substances differ from the so called bacterial hemotoxins in that they are contained within the bacterial cells and are only set free on the dissolution of the latter, but they may, nevertheless, have as great a pathological significance.

The first observations were made when studying the properties of a poison produced by dissolving washed pneumococci in dilute solutions of bile or sodium cholate.[1] The solution so obtained, which produces acute death in guinea pigs on intravenous injection, was also found to be hemolytic when added to an emulsion of red blood corpuscles in salt solution. At first this hemolytic property was thought to be due to the sodium cholate contained in the solution, but careful titration of the hemolytic power of the toxin showed that it was much greater than could be accounted for by the contained sodium cholate. In certain experiments, three hundred times as much sodium cholate in salt solution was required to produce hemolysis of 0.5 c.c. of an emulsion of sheep corpuscles as was contained in a minimal lytic dose of the toxin. It is not likely that the mere presence of bacterial substance increases the activity of cholate solutions, since the addition of protein to a cholate solution lessens its activity, and even peptone was found to have no intensifying action, but a slightly inhibiting action instead.

Later experiments have shown conclusively that cholate plays no important part in the reaction, since the lytic substances are present in extracts of pneumococci, obtained by allowing pneumococci to undergo autolysis in salt solution and also in extracts prepared by freezing and grinding the bacteria, in both cases without adding any cholate whatsoever.

PREPARATION OF THE TOXIN.

In the study of the lytic effect of substances obtained from the bodies of pneumococci, the extracts have been prepared in one of the following ways:

(1) Pneumococci are grown for twenty hours in broth, removed from the broth by centrifugalization, and washed once in 0.85 per

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[1] Cole, R., *Jour. Exper. Med.*, 1912, xvi, 644.

cent. salt solution. A test of the hemolytic power of toxins made from cultures grown for various lengths of time has shown that the toxins made from cultures twenty to twenty-four hours old are the most active. Toxins made from forty-eight-hour cultures possess little hemolytic power, while those made from seventy-two-hour cultures have no lytic power whatever. This difference is probably associated with the lysis of the bacteria which goes on in old cultures. The washed bacterial sediment is taken up in a small amount of salt solution, usually ten cubic centimeters for the bacteria from one liter of broth culture, and an amount of a 2 per cent. sodium cholate solution barely sufficient to cause solution of the bacteria is added. Usually one cubic centimeter is sufficient. After lysis has occurred, the solution is diluted with salt solution. If the bacteria were obtained from one liter of culture, the solution is usually made up to one hundred cubic centimeters. Different races of bacteria differ in the readiness with which they dissolve in cholate solution. Moreover, different solutions of bacterial bodies obtained in this way differ in their power to produce death in guinea pigs and also to produce hemolysis. Toxins, as above described, however, usually produce acute death in guinea pigs in doses of three to four cubic centimeters, and hemolysis of 0.5 of a cubic centimeter of sheep corpuscles in doses of 0.02 of a cubic centimeter or less, the whole mixture of toxin and corpuscles being made up to 2.5 cubic centimeters with salt solution.

(2) Pneumococci are grown in broth, washed, frozen, and ground, and the powder is dissolved in salt solution. A diluted toxin so prepared, which kills a guinea pig acutely in doses of three to four cubic centimeters, also usually produces hemolysis of sheep corpuscles, with the technique above described, in doses of 0.02 of a cubic centimeter or less.

Toxins prepared in the above ways are identical in their reactions so far as studied, and in the following pages no mention will be made of the method of preparation in each individual experiment.

A few experiments have also been conducted with extracts prepared by allowing pneumococci to undergo autolysis in salt solution. A series of tubes were prepared, all containing equal quantities of washed pneumococci and equal quantities of salt solution. These were kept at 37° C., and from time to time a tube was removed and the hemolytic power of the fluid tested. It was found that the hemolytic power of the emulsion rapidly increased and between six and eight hours was at its maximum. There was a very slight fall in hemolytic power up to eighteen hours, but at twenty-four hours it had markedly diminished and was entirely absent after forty-eight hours.

PROPERTIES OF THE TOXIN.

Toxins prepared by solution of pneumococci are lytic for rabbit, sheep, guinea pig, and human red blood corpuscles. Other corpuscles have not been tested. The hemolytic power is greatest for guinea pig corpuscles, less for sheep and human corpuscles, and least for rabbit corpuscles, but the differences are not striking. The rate of hemolysis depends upon the concentration of the toxin. If the concentration be sufficiently great, complete hemolysis may occur within five to ten minutes at 37° C.

Active toxins have been obtained from pneumococci belonging to all of the four immunological groups.[2]

An attempt has been made to discover whether or not any relationship exists between the virulence of organisms employed and the hemolytic power of the extract, and a larger number of races have been studied with this point in mind. Rosenow[3] has stated that the more virulent races of pneumococci autolyze most readily. This is generally true, but there are many exceptions. Also the more virulent races are more soluble in bile, though to this also there are apparent exceptions. When a series of cultures of pneumococci are tested, these two properties do not bear constant positions with reference to the virulence of the organisms. It may also be stated from our study that the most active hemolytic toxins are usually obtained from those races of pneumococci that have been most lately cultivated from the animal body and are most virulent. But here again there are sufficient exceptions to throw some doubt on the validity of the generalization. The attempt to increase the hemolytic power of the toxin produced from a given race by repeated passage through animals, testing the toxin production from time to time, does not yield results that are uniform and consistent. With a given race the hemolytic power of the toxin varies markedly from time to time, even though in each test the toxin is prepared in exactly the same way. This variation probably depends somewhat on the luxuriance of the growth, and this on many factors,—the exact composition of the medium, the exact temperature of the thermostat, etc. The amount of autolysis that has gone on in the culture before centrifugalization is also of importance. It is therefore impossible to predict with any given race and culture exactly the strength of the toxin that will be obtained. The conditions are quite different from those obtaining in the production of diphtheria toxin with different races of bacilli. Here races vary markedly in their power to produce toxin, but this property seems to be fixed in certain races, and no matter how long they are grown outside the body or under what unusual conditions, the power of producing toxin in large amounts is preserved.

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[2] Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.*, 1913, lxi, 727.

[3] Rosenow, E. C., *Jour. Infect. Dis.*, 1912, x, 113.

As regards the relationship between the hemolytic power and the toxic power as tested by intravenous injection into guinea pigs, there is a much more constant relationship. Even here the parallelism is not exact, but actively hemolytic solutions have always been found to be toxic and, with a few exceptions, the reverse is true. During the past two years toxins from a large number of cultures have been tested, and from the results obtained the above conclusions are derived.

The same manipulations which affect the hemolytic power of the toxin also affect the toxic power. It is impossible to draw an absolute conclusion from this that the same substance which produces hemolysis is the cause of the fatal effect in guinea pigs, though this is strongly suggested. On the other hand, symptoms and pathological changes in the guinea pigs do not seem to indicate that the animals die from the effects of hemolysis alone. In these animals hematuria frequently occurs and focal hemorrhages are seen post mortem, but there are no indications of a wide-spread hemolysis either when death occurs acutely or only after several hours. It is possible that, as in tetanus toxin, the effects may be due to two substances occurring together.

The effect of heat, acids, etc., on the toxin as tested by its power to produce hemolysis, corresponds with the previously reported effects of these agents on the poison as tested by injection into guinea pigs.[4] Heating to 55° C. for one half hour in our experience always destroys the hemolytic power of the toxin. Heating for one and one half hours at 45° C. usually has no effect, though in one experiment the hemolytic power was diminished after heating one half hour at 45° C. Rosenow[5] has stated that if the autolysate of pneumococci be plunged into boiling water and boiled for ten minutes, then quickly cooled in ice water, the toxicity for guinea pigs frequently remains. As far as the hemolytic effect is concerned, this statement does not hold good, for boiling for ten minutes and then plunging into ice water completely destroys its activity. The hemolytic power is slowly lost when the toxin is kept for some time on ice, though the change does not begin until after eighteen to twenty-four hours.

The hemolytic effect of the solution is markedly diminished or entirely lost after passing through a Berkefeld filter. However, even where controls have shown that the filters entirely prevent the passage of bacteria, the filtrate may still possess some hemolytic power, but it is diminished.

Digestion of the hemolytic toxin with trypsin destroys its activity within forty-five minutes. This is shown by the following experiment.

Fairchild's trypsin solution was employed, each cubic centimeter of which contains 300 units. Even dilute solutions of sodium hydroxide may cause hemolysis, so in making these tests a 5 per cent. solution of sodium carbonate was used to render the mixture alkaline.

The following mixtures were prepared, using toxin prepared from frozen and ground bacteria:

(*a*) 12 c.c. toxin.
3 c.c. 0.85 per cent. sodium chloride solution.
(*b*) 12 c.c. toxin.
1.5 c.c. trypsin solution.
1.5 c.c. 0.5 per cent. solution sodium carbonate.

Two series of tests were made (table I). In series 1 the mixtures were made up cold and the sheep corpuscles were added at once. In series 2 the mixtures were incubated at 37° C. for 45 minutes before the corpuscles were added.

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[4] Cole, R., *Jour. Exper. Med.*, 1912, xvi, 644.

[5] Rosenow, E. C., *Jour. Infect. Dis.*, 1912, xi, 235.

TABLE I.[6]

Series 1.

Tube No.	1 c.c. of mixture in dilution.	Sodium chloride solution.		Emulsion of sheep corpuscles.	Results. Readings after 3 hrs. at 37° C. Mixture (*a*).	Mixture (*b*).
1	1 : 2	1 c.c.		0.5 c.c.	+	+
2	1 : 4	1 c.c.		0.5 c.c.	+	+
3	1 : 8	1 c.c.		0.5 c.c.	+	+
4	1 : 16	1 c.c.		0.5 c.c.	+	+
5	1 : 32	1 c.c.		0.5 c.c.	+	+
6	1 : 64	1 c.c.		0.5 c.c.	+	+
7	1 : 128	1 c.c.		0.5 c.c.	±	±
8	1 : 256	1 c.c.		0.5 c.c.	ϕ	o
9	1 : 512	1 c.c.		0.5 c.c.	o	o
			Series 2.			
1	1 : 2	1 c.c.	At 37° C. for 45 min.	0.5 c.c.	+	o
2	1 : 4	1 c.c.		0.5 c.c.	+	o
3	1 : 8	1 c.c.		0.5 c.c.	+	o
4	1 : 16	1 c.c.		0.5 c.c.	+	o
5	1 : 32	1 c.c.		0.5 c.c.	+	o
6	1 : 64	1 c.c.		0.5 c.c.	+	o
7	1 : 128	1 c.c.		0.5 c.c.	+	o
8	1 : 256	1 c.c.		0.5 c.c.	±	o
9	1 : 512	1 c.c.		0.5 c.c.	±	o

Controls made with toxin plus sodium carbonate and with sodium carbonate alone showed that the latter had no effect on hemolysis.

The fact that the action of trypsin destroys the hemolytic effect of the solution obviously does not prove that the toxin is of protein nature. It indicates, however, that the toxin is probably closely associated with the protein constituents.

Attempts to extract the toxic substance with ether have so far proved unavailing. The toxin has been extracted with large amounts of ether, the ether evaporated under a fan in the cold, and the oily residue taken up in a small amount of alcohol and made into an emulsion in salt solution. Such an emulsion, however, has not been found hemolytic. There is no evidence, therefore, that the hemolytic effects are due to fatty or lipoidal constituents of the bacterial cells.

The presence of blood serum is known to inhibit the action of certain hemolytic toxins, not only bacterial toxins but others as well. Experiments with normal serum showed that the action of the pneumococcus hemolytic toxin is also inhibited to some extent by the presence of normal horse serum, to a somewhat greater extent by normal sheep and normal human serum, and to a still greater extent by normal rabbit serum. This antihemolytic effect is also possessed by dilute solutions of egg albumen. The effect of mucus contained in the sputum of a patient suffering from pneumonia was also tested. The mucus was shaken in salt solution; this mixture was added to the toxin and after one half hour at 37° C., sheep corpuscles were added. It was found that the mucus also had marked inhibiting power. Noguchi[7] has brought evidence to show that the inhibiting effect of normal blood serum for tetanolysin is due, in part at least, to the presence of cholesterin. It was therefore important to determine whether or not the hemolytic effect of the pneumococcus toxin was inhibited by the presence of cholesterin.

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[6] In the tables + indicates complete hemolysis; ± indicates partial hemolysis; ϕ indicates a trace of hemolysis; o indicates no hemolysis.

A protocol of one of the experiments to determine the effect of cholesterin in inhibiting hemolysis is given below.

Toxin.—June 17, 1914. Toxin was prepared by adding the washed bacteria from 500 c.c. of a 20-hour broth culture of pneumococcus A69 to 2.5 c.c. of salt solution plus 1 c.c. of a 2 per cent. solution of sodium cholate, placing the mixture at 37° C. for 15 minutes and then adding salt solution to 50 c.c.

Cholesterin Emulsion.—Cholesterin crystals were dissolved in a small amount of warm ether, and then sufficient warm sodium chloride solution was added, shaking constantly, to obtain a 1 per cent. emulsion. This was heated over a steam bath for 30 minutes to drive off the ether and filtered.

The experiments (table II) have shown conclusively that exceedingly small amounts of cholesterin are able to inhibit the action of the toxin. It is probable that the inhibiting effect of serum is also due to its cholesterin content. It is unnecessary to discuss the theoretical aspects of this phenomenon here, since the problem has been thoroughly considered by Noguchi and others in connection with the inhibition of tetanus hemolysis by serum. As they have concluded, this inhibition by cholesterin probably indicates that the lipoidal constituent of the red blood cell plays an important part in hemolysis.

It also seemed of importance to determine the effect of lecithin on the hemolytic action of this toxin. Kyes[8] has shown that the presence of lecithin increases the hemolytic action of cobra venom,

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[7] Noguchi, H., *Univ. Penn. Med. Bull.*, 1902, xv, 327.

[8] Kyes, P., *Berl. klin. Wchnschr.*, 1902, xxxix, 918.

TABLE II.

Hemolytic Test with Toxin.

Tube No.	Experiments.	Hemolysis.	
		1 hr. at 37° C.	24 hrs. on ice.
1	1 c.c. toxin undiluted + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
2	1 c.c. toxin diluted (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
3	1 c.c. toxin diluted (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
4	1 c.c. toxin diluted (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
5	1 c.c. toxin diluted (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
6	1 c.c. toxin diluted (1 : 32) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
7	1 c.c. toxin diluted (1 : 64) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+	+
8	1 c.c. toxin diluted (1 : 128) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	±	±

Test of Inhibition with Cholesterin.

1	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100)	30 min. at 37° C. 0.5 c.c. sheep corpuscles added to each tube.	0	0
2	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 500)		0	0
3	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 1,000)		0	0
4	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 5,000)		0	0
5	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 10,000)		0	0
6	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 50,000)		ø	±
7	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100,000)		±	±
8	1 c.c. toxin diluted (1 : 25) + 1 c.c. sodium chloride solution		+	+
1	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100)	5 min. at 37° C. 0.5 c.c. sheep corpuscles added to each tube.	0	0
2	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 500)		0	0
3	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 1,000)		0	0
4	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 5,000)		0	0
5	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 10,000)		0	ø
6	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 50,000)		ø	±
7	1 c.c. toxin diluted (1 : 25) + 1 c.c. cholesterin emulsion (1 : 100,000)		+	+
8	1 c.c. toxin diluted (1 : 25) + 1 c.c. sodium chloride solution		+	+

the lecithin acting as an activator. He[9] has also been able to form a combination of the active constituent of cobra venom with lecithin. On the other hand, it has been shown that with other hemolytic toxins lecithin may have an inhibiting action.

The following protocols of experiments indicate the effect of lecithin on the pneumococcus hemolytic toxin (table III).

TABLE III.

Tube No.	Experiments of July 2, 1914.		Hemolysis.
1	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 10)	30 min. at 37° C. 0.5 c.c. emulsion of sheep corpuscles added to each tube.	0
2	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 50)		0
3	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 100)		0
4	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. 1% lecithin emulsion diluted (1 : 500)		+
5	1 c.c. toxin A69 diluted (1 : 32) + 1 c.c. sodium chloride solution		+
6	1 c.c. toxin A69 diluted (1 : 64) + 1 c.c. sodium chloride solution		+
7	1 c.c. toxin A69 diluted (1 : 128) + 1 c.c. sodium chloride solution		+
8	1 c.c. toxin A69 diluted (1 : 256) + 1 c.c. sodium chloride solution		±

The tubes were kept for 1 hr. at 37° C. and 24 hrs. on ice.

The lecithin emulsion was prepared by making a 1 per cent. solution of Merck's lecithin in methyl alcohol, and of this a 10 per cent. emulsion was made in 0.85 per cent. salt solution.

This and other similar experiments have shown that lecithin in low dilutions has slight inhibiting action on the hemolytic effect of the toxin.

To learn whether non-hemolytic doses were rendered hemolytic by the presence of lecithin, experiments like the following were made (table IV).

From this and a number of similar experiments, it is evident that lecithin in no case increases the action of the hemolytic toxin. Except for the slight inhibiting action previously noted, therefore, lecithin has no effect on the hemolytic action of the toxin.

[9] Kyes, P., *Berl. klin. Wchnschr.*, 1903, xl, 956, 982.

TABLE IV.

Tube No.	Experiments of March 31, 1914.	Hemolysis.
1	1 c.c. toxin (1 : 10) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+
2	1 c.c. toxin (1 : 50) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	+
3	1 c.c. toxin (1 : 100) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	±
4	1 c.c. toxin (1 : 500) + 1 c.c. sodium chloride solution + 0.5 c.c. sheep corpuscles	0
5	1 c.c. toxin (1 : 500) + 1 c.c. lecithin emulsion (1 : 10) + 0.5 c.c. sheep corpuscles	0
6	1 c.c. toxin (1 : 1,000) + 1 c.c. lecithin emulsion (1: 10) + 0.5 c.c. sheep corpuscles	0
7	1 c.c. sodium chloride + 1 c.c. lecithin emulsion (1 : 10) + 0.5 c.c. sheep corpuscles	0

The tubes were kept for 2 hrs. at 37° C.

NATURE OF THE HEMOLYTIC TOXIN.

It is not the purpose of the present paper to discuss the chemical nature of the toxin or its mode of action. Sufficient study of it has not been made to render such a discussion profitable. From the properties already described it is evident that it is closely related to certain other vegetable and animal hemolytic toxins. It is extremely labile, is readily absorbed (as shown by the difficulty with which it passes through a bacterial filter), is destroyed by the action of trypsin, and its action is prevented by the presence of minute amounts of cholesterin and of larger amounts of lecithin.

To the view that it does not exist preformed in the bacterial cell, but is a product arising during self digestion or autolysis, the conclusive objection may be raised that it is present in the solutions prepared by freezing, drying, and grinding the bacteria. During this process there has been no opportunity for autolysis and the conclusion seems justified that the hemolytic substance is contained within the living bacterial cell.

ANTIHEMOLYTIC SERA.

In order to demonstrate that the hemolytic substance under discussion is of the nature of a true toxin, it is necessary to show that it possesses antigenic properties; that is, that its action is inhibited by the serum of animals immunized to it. For purposes of immunization rabbits and sheep were employed. These were injected intravenously with increasing doses of the toxin every seven to eight days. The toxins for injection were prepared by dissolving the pneumococci in sodium cholate solution, according to the method previously described, and they were centrifugalized before injection. It is probable that with each injection a few living organisms were also introduced. Since the serum of these animals acquired no agglutinating power for the homologous organisms, however, it is not likely that the acquired properties of the serum were due to the antigenic properties of these few bacteria.

(*a*) *Immune Rabbit Serum.*—The protocol of one experiment is given below (table V).

Rabbit 82–E.—Immunization commenced June 14, 1912. Received 5 doses of toxic extract intravenously during a period of 4 months. The animal received no further injections until June 5, 1913. It then received 7 doses of toxin intravenously at intervals of 6 to 7 days, the last injection being made on July 19. Bled on July 31.

Rabbit 285–A.—Immunization commenced June 12, 1913. Received 7 increasing doses of toxin intravenously, the last one being given on July 19. Animal bled on July 31.

TABLE V.

Sera Tested July 31, 1912. Toxin Prepared from Homologous Organisms.

Experiments.		Hemolysis.			
		Serum 82–E.	Serum 285–A.	Normal rabbit serum.	No serum.
Toxin diluted (1 : 8) + serum diluted (1 : 10)	½ hr. at 37° C.	0	0	±	
Toxin diluted (1 : 8) + serum diluted (1 : 50)		0	0	+	
Toxin diluted (1 : 8) + serum diluted (1 : 100)		0	0	+	
Toxin diluted (1 : 8) + serum diluted (1 : 500)		±	±	+	
Toxin diluted (1 : 8) + serum diluted (1 : 1,000)		±	+	+	
Toxin diluted (1 : 8) + sodium chloride					+

The experiments have been repeated many times with the sera of six rabbits immunized to this toxin. While in no case has the antihemolytic power of the immune serum been greater than 0.002 of a cubic centimeter to two hemolytic units of toxin, in all experiments the antihemolytic power of the immune serum has been considerably greater than that of normal rabbit serum. Tests of the rabbit sera for agglutinins were made from time to time, but none of the sera acquired the power of agglutinating the homologous organisms.

(*b*) *Immune Sheep Serum.*—Three sheep have been given increasing doses of extracts of pneumococci. Two of these animals were treated with extracts of pneumococci of type I and one received injections of pneumococci of type III, the so called *Pneumococcus mucosus.* The results obtained from the study of the sera of these sheep were identical, and data concerning the serum of but one are given.

Sheep A.—Immunization was commenced May 29, 1913. The toxins for injection were prepared by dissolving pneumococci in sodium cholate solution, as previously described, and the injections were all made intravenously. During the period of about 6½ months the sheep received 18 injections of the toxin. The first injection of the toxin was prepared from the bacteria contained in 12.5 c.c. of a twenty-four-hour bouillon culture. The injections were gradually increased in size. On Dec. 2, 1913, the toxin injected was prepared from the bacteria contained in 1,900 c.c. of a twenty-four-hour bouillon culture. On Dec. 10, an injection was made of toxin prepared from the bacteria contained in 3,000 c.c. of a twenty-four-hour bouillon culture. This injection was apparently too large and probably contained a considerable number of living organisms. Following this injection the animal appeared sick, the temperature was elevated, and the respirations were rapid. After a few days' illness the animal appeared better, but a cough persisted for 2 months with gradual loss of weight and strength, and it died Feb. 10, 1914.

Autopsy.—The pleural and pericardial sacs showed extensive fibrous adhesions. The lungs were edematous. Smears and cultures from the heart's blood showed the presence of pneumococci and a gas-forming anaerobic bacillus morphologically like *Bacillus tetani.* Unfortunately, through an error the cultures were destroyed before the identity of the latter organism could be accurately determined.

TESTS OF SERUM OF SHEEP A.

Agglutination.—The serum was repeatedly tested for agglutination with the homologous organism, the last test being made with serum obtained on December 2, 1913. At no time did the serum possess any agglutinating power.

Protection.—Repeated tests of the protective power of the immune sheep serum for mice were made, employing the technique used in this laboratory for determining protective power.[10] It was found that the serum possessed fairly well marked protective power against pneumococci of type I, and some protective power against pneumococci of other types, except type III, against which no protective power is ever present. The protective power, however, was never so high as that of the serum of horses immunized by injection of living organisms, nor was it so specific.

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[10] Dochez, A. R., *Jour. Exper. Med.*, 1912, xvi, 665.

TABLE VI.

Sheep Serum A, Obtained September 14, 1913. Inactivated. Tested September 15, 1913. Toxin 1.70.

Tube No.	Experiments.		Hemolysis.	
			Normal sheep serum.	Immune sheep serum A.
1	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 10) ...	½ hr. at 37° C. 0.5 c.c. emulsion sheep corpuscles added to each tube.	0	0
2	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 50) ...		ø	0
3	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 100) ..		ø	0
4	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 500) ..		±	0
5	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 1,000).		+	0
6	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 5,000).		+	±
7	1 c.c. toxin 1.70 + 1 c.c. serum diluted (1 : 10,000)		+	+
8	1 c.c. toxin 1.70 + 1 c.c. sodium chloride		+	+

The tubes were kept for 1 hr. at 37° C. and 24 hrs. on ice.

Antihemolytic Power.—(Table VI.) This and many similar experiments indicate that the serum of an animal immunized by the injection of toxin possesses an increased antihemolytic power. This antihemolytic action is not highly specific, however, as regards the type of organisms; a serum produced by the injection of toxin prepared from pneumococci of type I protects almost as well against a toxin prepared from organisms of type II as against one prepared from pneumococci of homologous type.

In immunization in this manner, the possibility cannot be excluded that in addition to the introduction of the toxin living organisms have also been introduced. It was therefore important to know whether or not an immune serum produced by the injection of living organisms would or would not have an effect on inhibiting the action of the hemolytic toxin. For this purpose the sera of horses immunized by the injection of living pneumococci were studied.

(*c*) *Immune Horse Serum.*—The horses from which these sera were obtained were immunized by injecting intravenously repeated and increasing numbers of living pneumococci previously washed in salt solution. These bacteria were obtained by centrifugalization from bouillon cultures. Usually one or two doses of pneumococci killed by heat were injected before proceeding to the injection of living bacteria. The sera studied were those that have been used in the treatment of patients and were active in protection only against the organisms of the type used in immunization. The sera were actively and specifically agglutinating.

Serum I had such protective power that when 0.2 of a cubic centimeter of the serum was mixed with 0.1 of a cubic centimeter of a twenty-hour bouillon culture of organisms of type I and the mixture was injected into a mouse, the mouse lived; whereas 0.000001 of a cubic centimeter of the culture injected alone killed within twenty-four hours.

Serum II had a little less protective power, in that 0.2 of a cubic centimeter of serum protected against only 0.01 of a cubic centimeter of culture of which 0.000001 of a cubic centimeter injected alone killed within twenty-four hours.

Tests of the antihemolytic power gave results similar to those obtained in an experiment of which the following is the protocol (table VII).

The study of these sera showed that they possessed high neutralizing power for the hemolytic poison obtained from the bodies of pneumococci, even higher than that present in the serum of rabbits or sheep injected with the toxin. This antihemolytic power, however, is not very specific as regards type of organisms, serum I protecting against the toxin prepared from organisms of type II almost as well as against that prepared from organisms of type I and *vice versa.* That this protective action is not merely a non-specific reaction of all immune sera, however, is shown by the fact that an anti-influenzal serum, kindly supplied by Dr. Wollstein,[11] possessed little or no greater antihemolytic power than did normal serum. In the experiment above described, the anti-influenzal serum had a little greater effect than did normal horse serum, but it was no greater than that of other normal horse sera tested at other times.

(*d*) *Antihemolytic Action of the Serum of Patients Sick of Pneu-*

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[11] Wollstein, M., *Jour. Exper. Med.*, 1911, xiv, 73.

TABLE VII.

Toxin 1.70 Prepared from Pneumococci of Type I.

Experiments of October 7, 1913.			Hemolysis.			
			Immune horse serum I.	Immune horse serum II.	Normal horse serum.	Anti-influenzal serum.
Toxin (2 hemolytic units) + serum 0.1 c.c.	½ hr. at 37° C.	Sheep corpuscles 0.5 c.c.	0	0	+	0
Toxin (2 hemolytic units) + serum 0.02 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	±
Toxin (2 hemolytic units) + serum 0.01 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	+
Toxin (2 hemolytic units) + serum 0.002 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	+
Toxin (2 hemolytic units) + serum 0.001 c.c.		Sheep corpuscles 0.5 c.c.	0	±	+	+
Toxin (2 hemolytic units) + serum 0.0002 c.c.		Sheep corpuscles 0.5 c.c.	+	+	+	+
Toxin A69, Prepared from Pneumococci of Type II.						
Toxin (2 hemolytic units) + serum 0.1 c.c.	½ hr. at 37° C.	Sheep corpuscles 0.5 c.c.	0	0	+	0
Toxin (2 hemolytic units) + serum 0.02 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	+
Toxin (2 hemolytic units) + serum 0.01 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	+
Toxin (2 hemolytic units) + serum 0.002 c.c.		Sheep corpuscles 0.5 c.c.	0	0	+	+
Toxin (2 hemolytic units) + serum 0.001 c.c.		Sheep corpuscles 0.5 c.c.	0	±	+	+
Toxin (2 hemolytic units) + serum 0.0002 c.c.		Sheep corpuscles 0.5 c.c.	+	+	+	+

The tubes were kept at 37° C. for 1 hr. and 24 hrs. on ice.

monia and of Those Convalescent from That Disease.—The sera of patients suffering with pneumonia and those of patients during convalescence from this disease have been tested for antihemolytic power against the pneumococcus toxin. It has been impossible, however, to demonstrate that these sera possess an increased antihemolytic action over the controls with normal human serum and the serum of patients suffering from other diseases.

From these studies of the antihemolytic action of immune sera, it is evident that by the injection into rabbits and sheep of a solution containing the bacterial substance of pneumococci, the serum of these animals acquires an increased power of inhibiting the hemolytic action of such a solution. This change in the serum occurs when the fluid injected consists of solutions of the bacterial bodies in sodium cholate or of solutions prepared by freezing the bacteria and grinding them in salt solution. The antihemolytic power of these sera is not so great, however, as that of sera produced by the injection of living organisms. The latter sera, however, possess marked agglutinating properties, while the former sera have no power of agglutination. While in the production of antitoxic sera the possibility of the injection of a few living organisms cannot be excluded, the lack of agglutinating power renders it extremely probable that the development of antihemolytic properties is due to the injection of the hemolytic substance, and that, therefore, this hemolytic solution possesses antigenic properties and may be considered a true toxin.

SUMMARY.

Solutions of the bodies of pneumococci, obtained by dissolving them in dilute solutions of sodium cholate, by permitting them to undergo autolysis, or by first freezing, drying, and then grinding in salt solution, are actively hemolytic for rabbit, sheep, guinea pig, and human red blood corpuscles. The substance on which this hemolytic property depends is very labile, much of its activity is lost on passing through a filter, and it is destroyed by the action of trypsin. In its properties it corresponds to the substance contained in such solutions which causes the death of guinea pigs on intravenous injection. Its activity is prevented by the presence of minute amounts of cholesterin.

Following the injection of this solution into rabbits and sheep, the sera of these animals acquire increased power of inhibiting its hemolytic action. It therefore possesses antigenic properties.

It may therefore be concluded that the bodies of pneumococci contain a toxin that is hemolytic for red blood corpuscles. This substance is not simply a product of autolysis but undoubtedly exists preformed in the bacterial cell. However, it is not given up to the surrounding fluid as long as the bodies of the bacteria are intact. It may therefore be considered a hemolytic endotoxin.

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THE PRODUCTION OF METHEMOGLOBIN BY PNEUMOCOCCI.*

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When pneumococci are grown in media containing blood or hemoglobin, the red color of the latter is changed to a greenish brown, as may be well seen in blood agar plates on which pneumococci are growing. This change is undoubtedly due to the formation of methemoglobin. Butterfield and Peabody[1] have demonstrated that methemoglobin is formed when pneumococci are cultivated in media containing blood.

A similar change in color, though not so pronounced, is seen in the blood of animals dying of acute pneumococcus septicemia, and to a still less extent in the blood of patients severely ill and dying of pneumonia. Peabody[2] has shown that during the terminal stages of fatal cases of pneumonia there occurs a progressive decrease in the oxygen content and the oxygen-combining capacity of the blood. This is evidently due to the transformation of oxyhemoglobin into methemoglobin. A similar change occurs in the blood of rabbits severely infected with pneumococci.[3] While the presence of methemoglobin in artificial culture media may be readily demonstrated by spectroscopic methods, this is more difficult in the blood of patients and infected animals, since a considerable concentration of methemoglobin is necessary for spectroscopic demonstration.

It has seemed of importance to learn more of the nature of the reaction during which methemoglobin is formed by pneumococci, and the present paper gives a report of this study, as far as it has been carried out.

Peabody stated that the transformation of oxyhemoglobin into

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* Received for publication, August 1, 1914.

[1] Butterfield, E. E., and Peabody, F. W., *Jour. Exper. Med.*, 1913, xvii, 587.

[2] Peabody, F. W., *Jour. Exper. Med.*, 1913, xviii, 7.

[3] Peabody, F. W., *Jour. Exper. Med.*, 1913, xviii, 1.

methemoglobin is brought about by the filtrates of pneumococcus cultures as well as by the cultures containing bacteria; but our experiments, which have been repeated many times, make it evident that in the experiments on which this statement was based faulty filters were employed. If the bacteria are all removed from a culture fluid, either by centrifugalization or by filtration through a Berkefeld filter, and then blood be added to the filtrate, no formation of methemoglobin occurs. The reaction therefore cannot depend merely upon the production of an acid reaction in the fluid in which the bacteria grow. This is also shown by careful neutralization of the culture before the addition of blood, when the rate of reaction is not changed unless to be increased.

Boiling a broth culture, or even heating it to 56° C. for one half hour, before addition of blood prevents the reaction from occurring. That this inhibition of the reaction by heat depends upon the destruction of the bacteria is rendered probable by the following experiment. A series of tubes containing broth cultures of different races of pneumococci were heated one half hour at 45° C.,—about the thermal death point for most races of pneumococci. Blood corpuscles were then added to each tube and transplantations were made from each tube on fresh media. In the cultures from which growth in the transplantation was obtained the formation of methemoglobin occurred. In the cultures, on the other hand, from which no growth occurred and in which the bacteria were therefore all killed, no formation of methemoglobin took place.

While extracts of pneumococcal bodies cause lysis of red blood corpuscles,[4] they never cause the formation of methemoglobin, unless they contain living pneumococci. The fact that small amounts of sodium cholate added to broth cultures of pneumococci inhibit the formation of methemoglobin might explain the failure of extracts of pneumococci in cholate solution to produce methemoglobin, but a similar failure to form methemoglobin is seen when the extracts are prepared by freezing and grinding the bacteria without the addition of cholate.

These experiments indicate that for the formation of methemoglobin the presence of living bacteria is necessary.

[4] Cole, R., *Jour. Exper. Med.*, 1914, xx, 346.

TABLE I.

Tube No.	Experiments.	Methemoglobin formation	
		1 hr. at 37° C.	1 hr. at 37° C. and 24 hrs on ice.
1	1 c.c. broth culture A8/0/6 + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
2	1 c.c. broth culture A8/0/6 (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
3	1 c.c. broth culture A8/0/6 (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
4	1 c.c. broth culture A8/0/6 (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
5	1 c.c. broth culture A8/0/6 (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
6	1 c.c. emulsion A + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
7	1 c.c. emulsion A (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+ in 20 min.	+
8	1 c.c. emulsion A (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+	+
9	1 c.c. emulsion A (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	+	+
10	1 c.c. emulsion A (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
11	1 c.c. emulsion B + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
12	1 c.c. emulsion B (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
13	1 c.c. emulsion B (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
14	1 c.c. emulsion B (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0
15	1 c.c. emulsion B (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles	0	0

It was now important to learn whether or not washed pneumococci in salt solution could produce the reaction or whether the presence of nutrient medium also is required. That the latter is necessary is shown by the protocol of an experiment which follows. Two portions of a twenty-hour broth culture of pneumococcus A8/o/6 were centrifugalized, the supernatant fluid was removed, and each sediment thoroughly washed in sodium chloride solution and again centrifugalized. One portion of the sediment was now made up to the original volume in broth (emulsion A), and the other portion was made up to the original volume in sodium chloride solution (emulsion B) and tests were made to determine the methemoglobin-producing power (table I) of the two solutions in varying dilutions, compared with the methemoglobin-producing power of an untreated broth culture.

It was suggested that the failure of the reaction to occur in salt solution might be due to the toxicity of sodium chloride in the absence of other inorganic salts. To test this, experiments were carried out with organisms suspended in Ringer solution. No reaction, however, occurred. It is evident, therefore, that some organic constituents of the broth are necessary for the reaction.

Experiments were then made to determine which class of organic substances in broth is essential. The following is a protocol of one such experiment (table II).

While experiments like those in table II showed that the reaction occurs when either sugar, peptone, or protein is present, further studies have shown that while protein substances must be present in quite high concentration in order that the reaction may occur, at least one part of a 10 per cent. solution of crystallized egg albumin to twenty parts of salt solution being required, the reaction occurs with great rapidity when sugar in very great dilution is present, even in dilutions as great as one part of a 5 per cent. dextrose solution to 10,000 parts of salt solution. The presence of traces of sugar in peptone solution and even in solutions of egg albumin can only with great difficulty be excluded, and it is possible that upon the presence of such traces of sugar the availability of these solutions in this reaction depends, since only extremely small amounts of sugar are required.

Various kinds of sugar were next tested to determine whether

TABLE II.

Tube No.	Experiments of December 13, 1913	Methemoglobin formation.	
		1 hr. at 37° C.	24 hrs. on ice.
1	1.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	0	0
2	1 c.c. sterile broth + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
3	1 c.c. sterile broth (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
4	1 c.c. sterile broth (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
5	1 c.c. sterile broth (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
6	1 c.c. sterile broth (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
7	1 c.c. 5% dextrose + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
8	1 c.c. 5% dextrose (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
9	1 c.c. 5% dextrose (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
10	1 c.c. 5% dextrose (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
11	1 c.c. 5% dextrose (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
12	1 c.c. 5% peptone + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
13	1 c.c. 5% peptone (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
14	1 c.c. 5% peptone (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
15	1 c.c. 5% peptone (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
16	1 c.c. 5% peptone (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
17	1 c.c. 4% egg albumin + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
18	1 c.c. 4% egg albumin (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
19	1 c.c. 4% egg albumin (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
20	1 c.c. 4% egg albumin (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
21	1 c.c. 4% egg albumin (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8	+ in 10 min.	+
22	1 c.c. sterile broth + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0
23	1 c.c. 5% dextrose + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0
24	1 c.c. 5% peptone + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0
25	1 c.c. 4% egg albumin + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution	0	0

The hemoglobin solution = 2 c.c. washed sheep corpuscles plus 8 c.c. distilled water plus 30 c.c. 0.85 per cent. sodium chloride solution.

Dextrose = 5 per cent. solution of chemically pure dextrose in distilled water, autoclaved for 20 minutes.

Peptone = 5 per cent. solution of Witte's peptone in distilled water, autoclaved for 20 minutes.

Egg albumin = 4 per cent. solution of crystallized egg albumin in distilled water.

Bacterial emulsion = emulsion in sodium chloride solution of washed sediment of 20-hour broth culture A8. The bacteria were in twice as great concentration as in the culture.

pneumococci cause the formation of methemoglobin only in the presence of dextrose or whether this sugar may be replaced by others in which the molecular configuration is different. Several races of pneumococci as well as streptococci were employed and the results are given in table III.

In another experiment *d*-xylose and *d*-arabinose were also tested. The results are given in table IV.

While in the experiments given no reaction occurred in the presence of inulin, in several other experiments a slight reaction occurred in solutions containing 2 per cent. of this sugar. In these and many other experiments as well, however, no reaction ever occurred in the solutions containing ribose. It is therefore apparent that while the reaction occurs in the presence of most sugars, the configuration of the molecule makes some difference. This is also seen in the effect of different sugars on the rate of reaction. The reaction always occurred more slowly with saccharose and arabinose than with the other sugars. Since the reaction occurs when pneumococci are placed in solutions containing traces of sugar, and it is known that pneumococci cause breaking down of many sugars, the possibility suggested itself that the reaction is due to some substance formed during the decomposition of the sugar molecule. The reaction cannot, however, be due simply to the formation of carbon dioxide. If carbon dioxide be passed through a solution of oxyhemoglobin the solution becomes cherry red, and if oxygen now be passed through it again becomes bright red. Such changes never occur in solutions of hemoglobin acted upon by pneumococci. A similar change is well seen when yeast is added to solutions containing blood corpuscles. In this case the blood takes on a magenta color which on shaking becomes bright red. It would appear possible, however, that the change might be due to some intermediate product of sugar metabolism. The exact transformation which the sugar molecule undergoes during the process of changing into carbon dioxide and water is still obscure, but there are several substances which are thought to represent intermediate stages. A series of such substances which are thought to be intermediate products in the metabolism of sugar and also certain ones that are known to be end products under certain conditions were tested. These substances

TABLE III.

Experiments.	Methemoglobin formation.			
	A18	P1.77	M1.77	Streptococcus.
1 c.c. 2% ribose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution......	0	0	0	0
1 c.c. 2% arabinose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 1 hr.	+ in 10 min.	+ in 1 hr.	0
1 c.c. 2% xylose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution......	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% maltose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.....	+ in 1 hr.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% lactose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.....	+ in 1 hr.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% saccharose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution ...	+ in 1 hr.	+ in 1 hr.	+ in 1 hr.	0
1 c.c. 2% dextrose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% levulose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% mannose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% galactose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution....	+ in 10 min.	+ in 10 min.	+ in 10 min.	0
1 c.c. 2% inulin solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution......	0	0	0	0
1 c.c. 2% raffinose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution	+ in 10 min.	+ in 10 min.	+ in 10 min.	0

The tubes were all kept at 37° C.

A18, P1.77, and M1.77 are pneumococci of type I. The streptococcus is a virulent hemolytic streptococcus.

TABLE IV.

Experiments.	Methemoglobin formation.		
	A19/5/7	A26/0/3	1/73
1 c.c. ribose. . . . . .2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0
1 c.c. *l*-arabinose. . . . . . .2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min.
1 c.c. *d*-arabinose. . . . . .2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0
1 c.c. *l*-xylose. . . . . .2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min.
1 c.c. *d*-xylose. . . . . .2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0
1 c.c. dextrose. . . . . .2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min.
1 c.c. mannose. . . . . .2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	+ in 15 min.	+ in 15 min.	+ in 15 min.
1 c.c. inulin. . . . . .2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution	0	0	0

The tubes were kept for 2 hours at 37° C.

were kindly supplied by Dr. G. M. Meyer. They were methylglyoxal, acetaldehyde, pyruvic acid, formaldehyde, formic acid, and acetic acid. Methylglyoxal, acetaldehyde, formaldehyde, and pyruvic acid produced methemoglobin only when very concentrated solutions were employed. The presence of bacteria did not increase their effect. Since such extremely small amounts of sugar are required in the formation of methemoglobin by bacteria, it is not possible that under these conditions any of the substances are present in a concentration sufficient to bring about the reaction, unless such substances are much more active in a nascent state than they are in solution.

The tests with formic acid showed that this substance caused the formation of methemoglobin only when it was used undiluted or but slightly diluted. Even under these conditions the reaction is due to the acidity of the solution, since, after neutralization and the addition of a mixture of primary and secondary potassium phosphates, in order to keep the mixture neutral, no reaction occurred, even with the undiluted acid. When a solution of formic acid, not in itself able to produce methemoglobin, is added to the bacterial emulsion plus hemoglobin solution, no formation of methemoglobin occurs, while the addition of a very dilute solution of dextrose under similar conditions causes the reaction to occur within a few minutes.

Similar results were obtained when acetic acid was used.

These studies, therefore, did not lead to the detection of any substance formed during sugar metabolism or fermentation to which the change of oxyhemoglobin into methemoglobin could be ascribed.

Other substances, such as alcohols, were also tested as to their effect in the formation of methemoglobin. The following results were obtained (table V).

None of these substances, except glycerin, when employed in low dilutions are able to replace dextrose in the mixtures in which methemoglobin is formed. When, however, glycerin, even in a dilution of 1 to 10,000, is added, the formation of methemoglobin occurs rapidly.

A large number of quantitative studies with these and similar substances were made, in the hope of finding some relationship in chemical structure between those substances which by their presence per-

TABLE V.

Experiments.	Results.
2 c.c. glycerin............(10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
2 c.c. mannite............(10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
2 c.c. methyl alcohol............(10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
2 c.c. ethyl alcohol............(10 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
1 c.c. bacterial emulsion + 1 c.c. glycerin............(1 : 10,000) + 0.5 c.c. hemoglobin solution	+ in 10 min.
1 c.c. bacterial emulsion + 1 c.c. mannite............(10 % solution) + 0.5 c.c. hemoglobin solution	+ in 3 hrs.
1 c.c. bacterial emulsion + 1 c.c. methyl alcohol............(10 % solution) + 0.5 c.c. hemoglobin solution	+ in 3 hrs.
1 c.c. bacterial emulsion + 1 c.c. ethyl alcohol............(10 % solution) + 0.5 c.c. hemoglobin solution	+ in 2 hrs.
1 c.c. bacterial emulsion + 1 c.c. dextrose............(5 % solution) + 0.5 c.c. hemoglobin solution	+ in 10 min.
1 c.c. bacterial emulsion + 1 c.c. starch............(1 % solution) + 0.5 c.c. hemoglobin solution	+ in 1 hr.
1 c.c. bacterial emulsion + 1 c.c. urea............(2 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
1 c.c. bacterial emulsion + 1 c.c. ethyl acetate............(0.2 % solution) + 0.5 c.c. hemoglobin solution	+ in 1 hr.
1 c.c. bacterial emulsion + 1 c.c. leucin............(2 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.
1 c.c. bacterial emulsion + 1 c.c. cholesterin............(1 % solution) + 0.5 c.c. hemoglobin solution	No change in 24 hrs.

mit pneumococci to form methemoglobin. So large a number of substances are suitable for the purpose that it has not been possible to show that any form of molecular configuration or grouping is essential.

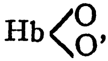
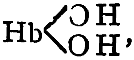
It seems probable, judging from these studies, that the formation of methemoglobin occurs whenever pneumococci in contact with hemoglobin are able to carry on metabolic and functional activities, probably including multiplication, and that this happens when traces of sugar and also when large amounts of other organic substances are present. While in the absence of those substances death of the bacteria does not necessarily occur, it is probable that their metabolic activities are reduced to a minimum.

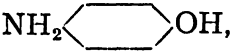
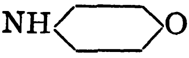
Cellular metabolic processes occur through the agency of ferments contained within the cells, and it would therefore seem probable, if the reaction under consideration were due to the active processes of oxidation occurring in the medium immediately surrounding the bacterial cells, that similar reactions would appear when bacterial extracts are used instead of the living bacteria. As Warburg[5] has pointed out, however, the reactions which occur in the living cell are not identical, at least in intensity, with those induced by the ferments when removed from the cell by extraction or other means. The activity of zymase, for instance, is markedly less than that of a corresponding number of living yeast cells. The importance of structure in cellular activity cannot be neglected, and from the fact that the reaction under consideration is not induced by the presence of bacterial extracts, it does not necessarily follow that the reaction is not due to the functional activities of the bacterial cells.

Studies concerning the formation of methemoglobin from oxyhemoglobin by means of chemicals indicate that the reaction is of the nature of an oxidation. The exact chemical nature of oxyhemoglobin as well as that of methemoglobin has not been absolutely determined. It is not even definitely known whether the methemoglobin molecule contains an amount of oxygen equal to or less than that of the oxyhemoglobin molecule. There is considerable evidence,[6] however, for the view that while in oxyhemoglobin the

[5] Warburg, O., Ueber die Wirkung der Struktur auf chemische Vorgänge in Zellen, Jena, 1913.

[6] von Reinhold, B., *Ztschr. f. physiol. Chem.*, 1913, lxxxv, 250.

oxygen is loosely combined as in an oxide, the formula being usually written  $Hb\langle{}^{O}_{O}$, in methemoglobin the oxygen is as in an hydroxide. Whether the molecule contains one hydroxyl group as in Hb–OH, or two hydroxyl groups as in  $Hb\langle{}^{OH}_{OH}$, is not determined, though there is experimental evidence for both points of view. In either case, however, methemoglobin would represent a lower stage of oxidation than oxyhemoglobin, and the transformation of oxyhemoglobin into methemoglobin would therefore be of the nature of a reduction.

On the other hand evidence has been presented by Heubner[7] to show that the formation of methemoglobin is always an oxidation process. As is well known, substances which are known to be oxidizing agents and also those considered to be reducing agents may bring about the transformation of oxyhemoglobin into methemoglobin. An explanation of this fact, which Heubner presents, is that the reducing agents are first oxidized, this oxidation occurring better in the presence of oxyhemoglobin, and then reduced, giving up their oxygen to form methemoglobin. It is of great interest that certain substances, such as aminophenol, , are able to convert much more hemoglobin into methemoglobin than could occur if the reaction were a simple molecular one. One molecule of aminophenol may apparently transform at least fifty molecules of hemoglobin into methemoglobin. The aminophenol must therefore repeatedly react with the hemoglobin. Heubner's view is that the aminophenol is first oxidized to a quinone, (quinonimine, ) and then again reduced to aminophenol, in this reaction the hemoglobin being changed to methemoglobin. The reaction may be repeated many times, the aminophenol therefore having a catalytic-like action.

If this be the true explanation of the kind of reaction occurring in the transformation of oxyhemoglobin into methemoglobin by organic chemical substances, it is possible that the mode of action of pneumococci in causing the formation of methemoglobin may follow similar lines. The metabolic activities of cells consist largely of oxidative and reduction processes. When oxyhemoglobin is brought into contact with pneumococci it may be changed into methemoglobin, because it is then exposed to active oxidative and reduction processes occurring in the neighborhood of the bacterial cells. These processes must be of a special type in the case of pneumococci, however, since otherwise all living cells would cause the transformation.

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[7] Heubner, B., *Arch. f. exper. Path. u. Pharmakol.*, 1913, lxxii, 239.

That this change goes on in the neighborhood of the cells and not necessarily in the cells themselves is shown by the fact that while the reaction occurs more readily when the hemoglobin is in solution in the medium surrounding the bacteria, it may also occur when the hemoglobin is contained within red blood corpuscles, the blood cells and bacteria being merely in intimate contact. If, however, the bacteria and blood cells are separated by a membrane of any kind, even a very thin layer of oil, the reaction does not occur.

If the change of hemoglobin into methemoglobin taking place in the neighborhood of pneumococcal cells is due to oxidative processes occurring there, it was thought that evidence for this fact would be obtained by studying the effect which the presence or absence of free oxygen would have on the reaction. This was done by testing the rate of reaction under the following conditions: To remove oxygen from a solution, a stream of hydrogen was passed through it for ten minutes, the entrance tube passing completely to the bottom of the tube containing the solution. The solution was then covered by a layer of paraffin oil. Passing hydrogen in this way through a solution of hemoglobin causes the solution to take on a dark color due to the formation of reduced hemoglobin. In mixing two solutions so treated, a pipette was passed through the paraffin layer of one to the bottom of the tube and the fluid was drawn into the pipette and added to the second solution, by plunging the tip of the pipette through the oil on the surface. Care was taken not to empty the pipette completely, as in this way air would be admitted. While complete absence of oxygen could not be obtained by this method, it was sufficiently excluded for the purpose intended.

It was found that if an emulsion of red blood cells, so treated with hydrogen, was added to a broth culture of pneumococci, also treated with hydrogen, no formation of methemoglobin occurred for some hours. When the tubes stood over night there usually occurred some change into methemoglobin, usually only at the surface under the layer of oil. If, moreover, such a mixture has stood for some time without the formation of methemoglobin, and oxygen now be bubbled through, the change into methemoglobin occurs with great readiness. It is evident, therefore, that the presence of oxygen is necessary for the reaction.

On the other hand, an excess of free oxygen somewhat delays the reaction. If two tubes are prepared, each containing a mixture of an emulsion of red blood cells and an emulsion of pneumococci, and if oxygen be bubbled through one tube, while the other is simply allowed to stand exposed to the air, the two tubes being kept at the same temperature, the change into methemoglobin occurs more slowly in the tube through which oxygen is passing. To control the possibility that the mechanical disturbance due to the bubbling gas may account for the difference, air was passed through the second tube. The reaction was again delayed in the tube through which oxygen was passing.

Two tubes were now prepared, one tube containing a mixture of an emulsion of red blood cells and an emulsion of bacteria, each of which had been previously saturated with hydrogen, and the other containing an identical mixture except that no hydrogen had been passed through. Oxygen was now bubbled through both tubes, which were kept at the same temperature and under identical conditions. In the tube containing the mixture previously saturated with hydrogen the reaction occurred more rapidly than it did in the other tube.

The above results are briefly shown in the following protocol of one experiment (table VI).

It is now easy to interpret these experiments in the light of

TABLE VI.

Tube No.	Experiments.	Methemoglobin formation.
1	4 c.c. broth culture A82/1/12 + 2 c.c. hemoglobin solution.......	+ in 7 min.
2	4 c.c. broth culture A82/1/12 saturated with hydrogen + 2 c.c. hemoglobin solution saturated with hydrogen	0
3	4 c.c. broth culture A82/1/12 + 2 c.c. hemoglobin solution } Oxygen bubbled through	+ in 28 min.
4	4 c.c. broth culture A82/1/12 saturated with hydrogen + 2 c.c. hemoglobin solution saturated with hydrogen } Oxygen bubbled through	+ in 17 min.

The tubes were all kept at 37° C.

what is known concerning the mode of production of methemoglobin by substances like aminophenol. According to this interpretation the formation of methemoglobin by pneumococci occurs as the result of reduction and oxidative processes occurring in the neighborhood of the bacteria. The oxyhemoglobin is first reduced and if this is inhibited by an excess of free oxygen the reaction is delayed. On the other hand, after reduction has occurred a free supply of oxygen accelerates the reaction. If oxygen be excluded no reaction whatever can occur.

The writer realizes that with the present knowledge it is impossible to conclude that precisely this mode of reaction occurs; but it seems to be the best explanation at present available of the observed experimental facts. It may be objected that the effect of the presence of hydrogen and oxygen is to inhibit or accelerate the metabolic activities of the bacteria rather than to cut off or to increase the supply of oxygen required for the chemical changes. The fact that an excess of oxygen delays the reaction is against this interpretation, though it is known that an excess of oxygen may inhibit cellular action or even be directly toxic to cells.

Experiments were also undertaken to determine whether, in the absence of free oxygen, the oxygen required for the reaction could be obtained from methylene blue, if this be added to the mixture. It was found that the reaction proceeds under these circumstances, but more slowly and less completely than in the presence of free oxygen.

It is believed that the experiments concerning the production of methemoglobin by pneumococci are important not only because they may possibly explain a reaction which probably occurs in every animal severely infected with pneumococci, but they are also important because they suggest a possible explanation for the pathological action of those bacteria which apparently do not produce an active toxin. Since bacteria may injure red blood corpuscles by merely changing oxidative processes in their vicinity, and without producing substances capable of isolation, it is possible that bacteria may injure other tissue cells in a similar manner. Therefore, the pathological effects of bacteria are not necessarily due to the action of a definite poison, but may be due to disturbances in oxidation in the immediate neighborhood of the bacteria.

CONCLUSIONS.

1. Pneumococci in contact with hemoglobin transform this into methemoglobin. This reaction occurs only when the pneumococci are living; it is not induced by the culture fluid or by extracts of the bacteria.

2. The reaction does not occur when hemoglobin is added to an emulsion of washed pneumococci in salt solution. However, if minute traces of dextrose be added to such a mixture, the reaction quickly occurs. The dextrose may be replaced by any one of a number of other sugars, and also by certain other organic substances, if the latter are added in large amounts. Certain other organic substances are not able to replace dextrose, but it has been impossible to determine any special molecular configuration on which this property depends.

3. The formation of methemoglobin by pneumococci probably resembles the formation of methemoglobin by certain chemical substances, such as aminophenol.

4. From the work of others it is probable that the formation of methemoglobin is always a reaction of oxidation. In the formation of methemoglobin by reducing agents, the latter are first oxidized, this occurring better in the presence of oxyhemoglobin. In certain instances an alternate oxidative and reduction of the transforming agent occurs, so that the reaction is continuous.

The effect which the presence or absence of free oxygen has on the reaction with pneumococci suggests that this follows similar lines.

5. The reaction does not occur in the absence of oxygen. If the free oxygen be first removed, and then replaced, the reaction occurs more rapidly than if the oxygen had not been removed. The presence of free oxygen in excess slightly delays the reaction, possibly because of the inhibition of the reduction process which forms the first part of the reaction.

6. The explanation of this phenomenon of methemoglobin production is not only of importance so far as this special reaction is concerned, but also because it suggests an explanation for the manner in which pathological effects are produced by those bacteria which apparently produce no soluble toxin.

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